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(57) Abstract

The present invention relates to methods of detection of cellular exposure to an addictive drug and methods for identifying a substance that can alter or mimic the cellular effects of an addictive drug. Some of the methods involve determining the localization of PKA subunits and PKC isozymes in cells exposed to an addictive drug such as ethanol.

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DETECTION OF CELLULAR EXPOSURE TO ADDICTIVE DRUGS

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Serial No. 09/311,206, filed May 12, 1999, which is incorporated herein by reference.

INTRODUCTION

Technical Field

The present invention is in the field of biological assays for the detection of exposure in mammals to ethanol or other addictive drugs and for the screening for compounds that modify the cellular effects of such exposure.

Background

The abuse of ethanol and other addictive drugs remains a major public health problem in the U.S. and throughout the world. It is of interest to provide methods for the detection of exposure in individuals to ethanol or other addictive drugs so as to monitor compliance with abuse treatment regimens. It is also of interest to provide assays useful for the evaluation of drugs that may be used to treat one or more of the adverse effects of chronic over-consumption of ethanol or other addictive drugs. In order to provide such methods and assays, it is necessary to gain detailed understanding of the biochemical effects of ethanol exposure at a cellular level. Recent evidence suggests that ethanol modifies the function of certain proteins and signal transduction pathways, thereby producing changes in second messenger concentrations, the activity or location of protein kinases, and gene expression. This observation does not provide a specific test which enables the effects of ethanol to be readily determined. Recently, it has been shown that the specificity of protein kinases appears to correlate with their localization within the cell (Mochly-Rosen, *Science* (1995) 268:247).

Chronic alcoholism causes functional and pathologic changes in many organs, particularly the brain, but the molecular mechanisms which account for these effects are not well understood. It would be desirable to provide methods for monitoring the effect of chronic ethanol exposure on mammalian cells, and further desirable to provide methods for determining if an individual has been actively consuming ethanol for extended periods of time.

The present invention relates to assays for detecting the effects of ethanol or other addictive drugs, particularly the chronic exposure of ethanol or other addictive drugs, on animal cells, particularly human or other mammalian cells. These assays can be used both in a diagnostic test to determine consumption of ethanol or other addictive drugs in an individual, or in screening for drugs or treatments which moderate, inhibit, reverse or enhance the effects of such consumption.

SUMMARY OF THE INVENTION

The present invention relates, in part, to the discovery that exposure to ethanol alters dramatically the subcellular localization of the catalytic Cα subunit and the RIα and RIIβ regulatory subunits of the cAMP dependent protein kinase (PKA) and the δ- and ∈-isozymes of protein kinase C (PKC). Exposure to other addictive drugs alters the subcellular localization of the catalytic Cα subunit of PKA, δ-PKC and ∈-PKC and is likely to alter the subcellular localization of other proteins as well. For example, the catalytic Cα subunit of PKA, which is normally localized to the Golgi apparatus area, appears to translocate to the nucleus upon exposure of a cell to ethanol. Ethanol also has been shown to cause translocation of PKC activity from cytosolic to membrane fraction in astroglial cells and human lymphocytes and epidermal keratinocytes. The present invention further relates to the discovery that the detectable amount of the regulatory subunit RI of PKA decreases, and the amounts of the α-, δ- and ∈-subunits of PKC increase in certain cell types, including but not limited to, NG108-15 cells (α-, δ-, and ∈-subunit) or PC12 cells (δ- and ∈-subunit), upon the exposure to ethanol. These discoveries provide the basis for assays that may be used to detect the exposure of cells to ethanol or other addictive drugs and further for assays that may be used for the screening of drugs or treatment to modulate the effects of consumption of ethanol or other addictive drugs.

One aspect of the invention is to provide assays that provide an indication of the exposure of a cell or an individual to an addictive drug by identifying at least one cell component, *e.g.*, a protein, that has a cellular localization (distribution) that varies in correlation with the exposure of the cell to the addictive drug, and determining the distribution of that cell component within a cell of a sample to be tested. In one preferred embodiment, the cell component comprises a subunit of the cAMP dependent protein kinase, PKA, the Cα subunit being particularly preferred. In another preferred embodiment, the cellular component comprises an isozyme of protein kinase C, PKC, wherein the δ or the ∈ isozyme of protein kinase C is particularly preferred. The invention also provides assays for determining chronic exposure to an addictive drug and methods for determining whether a mammal has been chronically consuming an addictive drug.

Another aspect of the invention is to provide assays that provide an indication of exposure of a cell or an individual to an addictive drug by measuring the amount of protein that varies in amount in a relationship with the exposure of the cell to the addictive drug. In one preferred embodiment, the decrease of the detectable amount of the regulatory subunit RI of PKA in response to exposure is determined. In another preferred embodiment, the increase of the detectable amounts of α-PKC, δ-PKC, or ∈-PKC in response to exposure is measured.

Another aspect of the invention is to provide assays for screening therapeutic compounds that modulate or mimic the effects of an addictive drug on a cell. Some of these screening assays measure the ability of a compound of interest to interfere with one or more of the cellular effects of an addictive drug described herein, *e.g.*, changes in the localization of PKA Cα, PKA RIα, PKA RIIβ, δ-PKC, or ∈-PKC, decreases in the amount of RI, increases in the amount of αPKC, δ-PKC, or ∈-PKC, changes in the set of proteins phosphorylated by or expressed in response to Cα, δ-PKC and ∈-PKC. For example, Cα may induce phosphorylation of (cAMP response element binding protein) CREB (cAMP response element binding protein) and thereby its activation, resulting in the induction of gene expression regulated by the cyclic AMP response element (CRE).

Another aspect of the invention is to provide kits for detecting the exposure of cells to an addictive drug or for identifying a substance that alters or mimics the effects of an addictive drug. Kits of the invention may include labeled antibodies

capable of specifically binding to Cα, RIα and RIIβ subunits of PKA or to the α-, δ- or ∈-isozymes of PKC.

Yet another aspect of the invention is to provide methods for reducing consumption of an addictive drug comprising administering a substance which alters or mimics the effects of the addictive drug on the cellular localization of, *e.g.*, PKA Cα, PKA RIα, PKA RIIβ, δ-PKC, or ∈-PKC.

Another aspect of the invention is to provide methods for reducing consumption of an addictive drug comprising administering two substances that modulate a receptor for the addictive drug and a factor that acts synergistically with the receptor in the alteration of the sub cellular of these proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1D show micrographs indicating the location of the PKA catalytic subunit staining in NG108-15 cells after a forty eight (48) hour exposure to 200 mM ethanol. The images in FIG. 1A and 1B were made with a confocal microscope, whereas those in FIG. 1C and 1D were made with a light microscope with a fluorescein filter, as were the inserts shown in FIG. 1A and FIG. 1B. FIG. 1A shows control cells in which majority of staining exists in the perinuclear Golgi area. FIG. 1B shows test cells exposed to ethanol, for which the staining is primarily within the nuclear region. FIG. 1C shows the reversibility of the distribution of staining upon withdrawal of ethanol from the cells. FIG. 1D shows cells labeled with an anti-Cα antibody solution that had been preabsorbed by purified Cα. No staining resulted, indicating that the stain is specific for Cα.

FIG. 2 is a graph showing the dependence of the percentage of cells exhibiting nuclear staining as compared to Golgi staining on the concentration of ethanol to which the cells have been exposed.

FIGS. 3A-3D are a series of micrographs comparing the movement of the Cα subunit of PKA in cells exposed to ethanol and that of cells exposed to various other agents as indicated.

FIG. 4 shows the variation of the percentage of cells with Golgi staining over time, for cells exposed to ethanol, and for cells treated with forskolin.

FIGS. 5A-5B show Western blot analysis of the Cα and RI PKA subunits in ethanol-exposed NG108-15 cells.

FIGS. 6A-6C depict immunohistochemical staining of δ-PKC in NG108-15 cells grown in defined medium in the presence or absence of EtOH or of PKC activation by phorbol myristate acetate (PMA).

FIGS. 7A-7C depict immunohistochemical staining of δ-PKC in NG108-15 cells after a four (4) day exposure to 25 mM ethanol. FIG. 7A-7C depict, respectively: control, non-ethanol exposed cells; test cells exposed to 25 mM ethanol; and a control showing the specificity of the staining when the anti-δ antibody is preabsorbed ("Pre.") with the immunizing peptide before labeling of the cells.

FIGS. 8A-8C depict immunohistochemical staining of ∈-PKC in NG108-15 cells grown in defined medium in the presence or absence of EtOH or of PKC activation by PMA.

FIGS. 9A-9C depict immunohistochemical staining of ∈-PKC in NG108-15 cells after four (4) day exposure to 25 mM ethanol. FIG. 9A-9C depict. respectively: control, non-ethanol exposed cells; test cells exposed to 25 mM ethanol; and a control showing the specificity of the staining when the anti-ε antibody is preabsorbed ("Pre.") with the immunizing peptide before labeling of the cells.

FIGS. 10A-D depict immunohistochemical staining of PKA RIα in human neutrophils isolated from nonalcoholics and alcoholics. FIG. 10A depicts untreated neutrophils from nonalcoholics, FIG. 10 B depicts untreated neutrophils from alcoholics, FIG. 10C depicts neutrophils from nonalcoholics after *in vitro* exposure of the cells to 200 mM ethanol for 30 minutes, and FIG. 10D depicts neutrophils from alcoholics after *in vitro* exposure of the cells to 200 mM ethanol for 30 minutes.

FIGS. 11A-B depict immunohistochemical staining of PKA Cα in human lymphocytes isolated from nonalcoholics and alcoholics, respectively.

FIG. 12 depicts CRE-regulated luciferase expression in transfected cells exposed to ethanol.

FIG. 13 depicts relative CRE-mediated luciferase activity in the presence (filled squares) or absence (empty squares) of 200 mM ethanol at the indicated times.

FIG. 14 depicts percent increase over control of CRE-luciferase activity after a 4hour and a 14hour exposure to 200 mM ethanol in the presence of BW A1434U (10μM) or Rp-cAMPS (20μM).

FIG. 15 depicts percent increase over ethanol-unexposed control of CRE-luciferase activity after a 14 hour exposure to ethanol in cells (a) pretreated for 30 min

in the absence or presence of the PKA inhibitor H-89 (10 μM.), 5μM KN-62, 2μM PD98059, 1μM U0126 or 50nM bisindolylmaleimide I (GF) or (b) co-transfected with a dominant negative DN-RIα or DN-MEK construct.

FIG. 16A depicts relative CRE-luciferase activity in cells exposed to ethanol for 14 hours and co-transfected with constructs expressing dominant negative CREB (CREB M1). FIG. 16B depicts percent increase in CRE-luciferase activity over ethanol-unexposed control in cells that express the Gal4-CREB fusion protein and Gal4-luciferase. Such cells were pretreated in the absence or presence of 20μM Rp-cAMPS for 2 h or with 5μM KN-62 or 50nM GF for 30 min. and were further incubated with ethanol for 14 hours.

FIG. 17 depicts a model for the interaction of the pathways triggered by treatment with ethanol or dopamine agonists.

FIG. 18 depicts western blot analysis of the localization of the Cα and Cβ catalytic subunits of PKA and the RI and RIIβ regulatory subunits of PKA in NG108-15 cells grown in the presence or absence of ethanol.

DESCRIPTION OF SPECIFIC EMBODIMENTS

I. DEFINITIONS

As used herein, the following term(s), whether used in the singular or plural, will have the meanings indicated:

An "addictive drug" is a substance, which, when consumed in quantities beyond a threshold level, creates for the consuming animal a compulsive need to consume more of the substance. Addictive drugs include, but are not limited to, ethanol opioids (*e.g.*, heroin), cannabinoids (*e.g.*, marijuana), nicotine, cocaine, addictive drugs that mediate agonist activity at the dopamine D2 receptor, and the like.

An "ethanol indicative protein" is a protein whose subcellular location or detectable amount is different in a cell exposed to ethanol compared to a cell that is not exposed to ethanol. Similarly, a "drug indicative protein" is a protein whose subcellular location or detectable amount is different in a cell exposed to an addictive drug compared to a cell that is not exposed to such addictive drug.

An "ethanol-exposed cell" ("EEC") is a cell which is or has been contacted with exogenous ethanol. An "ethanol-unexposed cell" ("EUC") is a cell which has not been contacted with exogenous ethanol. By "exogenous ethanol" is meant ethanol which has been introduced from outside a cell or organism. For example, exogenous ethanol can be ethanol added to a sample (*e.g.*, a cell culture) or ethanol consumed by a mammal from which a sample is obtained. Similarly, a "drug-exposed cell" ("DEC") is a cell which is or has been contacted with an exogenous addictive drug, and a "drug-unexposed cell" ("DUC") is a cell which has not been contacted with an exogenous addictive drug.

"Cellular localization" of an ethanol indicative protein refers to the cellular subregion in which the protein is present. A protein is "present" in a cellular subregion if it can be detected in the cellular subregion by any of the techniques known in the art and discussed herein.

A "cellular subregion" is a region within the cell that is detectably distinct from another region of the cell. Examples of cellular subregions include, but are not limited to, the nucleus, the perinucleus, the Golgi apparatus, and the cytoplasm. The "cytoplasm" is the region of the cell outside the perinucleus and can include structures present in the cytosol, such as, for example, the endoplasmic reticulum, mitochondria, lysosomes, peroxisomes, vacuoles, other cytoplasmic organelles, and other structures such as cytoskeletal filaments, etc. In smaller cells, such as lymphocytes, the Golgi apparatus may not be distinguishable from the rest of the cytoplasm. However, in larger cells such as NG108 cells, the Golgi apparatus and some of the structures described above may be visually distinguishable from the rest of the cytoplasm.

An "origin cellular subregion" is a cellular subregion in which a given ethanol indicative protein is predominantly present in an EUC. A "destination cellular subregion" is a cellular subregion in which an ethanol indicative protein is predominantly present in an EEC. In certain cells, some proteins may translocate to an intermediate destination cellular subregion and complete translocation to the destination cellular subregion as the duration of exposure increases. A "first cellular subregion" is typically the cellular subregion first discussed in a paragraph or a claim of this application. Depending on the context of the discussion, a first cellular subregion may be an origin cellular subregion or a destination cellular subregion. The phrase may also describe a plurality of cellular subregions where a protein is localized in more than one cellular subregions in a test cell and one or more cellular subregions

in a control cell, or vice versa. For example, δ-PKC is predominantly present in the Golgi apparatus of EUCs and in both the perinucleus and nucleus of EECs. Thus, the nucleus is a destination subregion for δ-PKC and the perinucleus is also a destination subregion for δ-PKC.

A protein is "predominantly present" in a cellular subregion if a detectably greater amount of the protein is present in that cellular subregion compared to another cellular subregion.

A "detectably different amount (or number)" refers to a difference in an amount or number that can be detected when comparing cellular subregions within a cell, or when comparing cells, such as comparing a test cell(s) and a control cell(s). A detectably different amount means that more can be detected in one cellular subregion vs. another or in one cell compared to another. One can quantitate the amounts if desired, but quantitation is not necessary to practice many methods of the invention. Preferably, the difference is a statistically significant difference, more preferably a difference greater than about 5%, even more preferably a difference greater than about 25%, with a difference of greater than about 35% up to 100% being most preferred.

A substance that "mimics the effects of an addictive drug" on cellular localization affects the cellular localization of a protein in a similar manner as does the addictive drug. For example, if the amount of the protein in a particular cellular subregion is (1) detectably greater in a DEC than a DUC, then it is detectably greater in a cell exposed to the substance than in a DUC; or (2) detectably less in a DEC than a DUC, then it is detectably less in a cell exposed to the substance. The amount of protein in the cellular subregion of a DEC need not be the same as the amount of protein in the corresponding cellular subregion of a cell exposed to the substance. Preferably, the amount of protein in the cellular subregion of a cell exposed to the substance differs from that in a DUC by a statistically significant amount, more preferably by more than about 5% and even more preferably approaches the amount of protein present in the cellular subregion of a DEC. For example, as discussed above, PKA Cα is present in a detectably greater amount in the nucleus of an EEC than in the nucleus of an EUC. Likewise, PKA Cα would also be present in a detectably greater amount in the nucleus of a cell exposed to a substance that mimics the effects of ethanol than in the nucleus of an EUC.

By "prior exposure" to ethanol or other addictive drug is meant that a sample has been exposed to exogenous ethanol before a particular point in time, such as, for example, before testing for such exposure. Usually the sample has been exposed at most two weeks before testing, preferably less than a week, even more preferably within 48 hours before testing. An example of prior exposure is found in a sample obtained from a mammal that has a detectable blood alcohol level. However, the exposure need not be continuous and it need not occur immediately before testing. For example, many alcoholics have blood alcohol levels close to or equal to zero in the morning. Thus, the phrase "prior exposure" includes chronic and/or episodic exposure.

By "chronic exposure" to ethanol or other addictive drugs is meant that a sample has been exposed to exogenous ethanol chronically before a particular point in time. The sample might not have been exposed immediately before testing is performed, or even within 48 hours before testing, but it has been exposed on a recurrent or prolonged basis for a time sufficient for the cellular effects of such exposure to be detectable whether or not the addictive drug is present in the sample at a detectable level. An example of chronic exposure is found in a sample obtained from a mammal that has been chronically consuming alcohol whether or not the mammal has a detectable blood alcohol level at the time the sample is obtained.

II. INTRODUCTION

The subject invention relates to discoveries concerning the effects of ethanol or other addictive drugs on the cellular localization and abundance of specific proteins as a consequence of exposure of cells to ethanol or other addictive drugs. Specifically, the invention relates to the discoveries that some proteins are predominantly present in different cellular subregions in cells exposed to ethanol or other addictive drugs compared to cells that have not been so exposed. Such proteins include, but are not limited to, the Cα catalytic subunit of cAMP-dependent PKC (PKA Cα), the RIα regulatory subunit of PKA (PKA RIα), the RIIβ regulatory subunit of PKA (PKA RIIβ), the α-isozyme of protein kinase C (α-PKC), the δ-isozyme of PKC (δ-PKC), and the ∈-isozyme of PKC (∈-PKC). While not wishing to be held to a particular theory, the inventors believe that the exposure of certain cells to addictive drugs induces translocation of such proteins from one cellular subregion

to another. For example, in EECs of some cell types, (1) the Cα catalytic subunit of cAMP-dependent protein kinase (PKA) (also referred to herein as PKA-Cα) moves from the Golgi region to the nucleus; and (2) the RIα regulatory subunit of PKA (also referred to herein as PKA-RIα) moves from the cytoplasm to the nucleus. It is also believed that in certain cells exposure of cells to ethanol induces translocation of the δ-isozyme of PKC from the Golgi region to the perinucleus and the nucleus, while inducing translocation of the ∈-isozyme of PKC from the perinucleus to the cytoplasm. As discussed in greater detail below, some of these proteins are localized differently in various cell types and some proteins do not translocate in some cell types. The localization of some proteins useful in the methods of the invention are shown in Table 1.

Additionally, the invention relates to the discovery that the detectable amount of type I (RI) regulatory subunit of PKA found in cells decreases in response to ethanol exposure, while the detectable amounts of α-PKC, δ-PKC, and ∈-PKC increase in response to short term as well as long term exposure to ethanol.

The cellular changes in response to exposure to ethanol or other addictive drugs have numerous consequences beyond the effects of ethanol on cellular localization of Cα and δ-PKC and ∈-PKC. For example, the dissociation of the catalytic subunit Cα from the regulatory subunit frees the Cα subunit to phosphorylate proteins. Furthermore, upon translocating to the nucleus, the Cα subunit of PKA may phosphorylate a different set of proteins than those available in the Golgi apparatus or elsewhere in the cytoplasm. Moreover, the translocation of the Cα subunit may also alter the extent of phosphorylation of different proteins phosphorylated by Cα, such as CREB, as well as it may alter the CRE-regulated gene expression. The Cα-mediated changes in protein phosphorylation may also have detectable effects on gene expression; such PKA effects may also be used to monitor ethanol exposure. Similar effects of δ-PKC and ∈-PKC translocation may be determined. Generally, proteins whose cellular localization or detectable amount are altered by ethanol exposure are referred to herein as ethanol indicative proteins.

The methods of the invention may be employed to monitor withdrawal of ethanol or other addictive drugs from a subject. Specifically, after a chronic alcoholic withdraws from alcohol, it is expected that, in the cell types in which altered localization occurs, Cα and RIα will leave the nucleus and return to the cytoplasm. In

some instances, the observed protein will physically return to the origin subregion. Usually, however, the observed protein remains in the destination subregion in the ethanol-exposed cells. But, in newly synthesized, ethanol-naive cells, the protein will be present in the origin subregion, giving the appearance of having "left" the destination subregion. Thus, the techniques described may be used to monitor the withdrawal from ethanol. Aspects of the invention discussed below focus on ethanol as the addictive drug. However, the methods of the invention are readily applicable to other addictive drugs. Exposure to such addictive drugs alters the subcellular localization of a cellular protein. Addictive drugs other than ethanol suitable as subjects for the methods described herein include, but are not limited to, opioids (*e.g.*, heroin), cannabinoids (*e.g.*, marijuana), nicotine, cocaine, addictive drugs that mediate agonist activity at the dopamine D2 receptor, and the like.

III. Methods Relating to Alteration of Subcellular Location of a Protein in a Cell Exposed to an Addictive Drug

In the embodiments described below, methods are explained for examining the effects of ethanol and other addictive drugs and screening for therapeutic agents which affect these effects either *in vivo* or *in vitro*. It will be readily apparent to one skilled in the art that these techniques may be applied to a number of problems.

The invention is not intended to be limited to the detection of the proteins mentioned above, but one skilled in the art may use other proteins whose behavior in the presence of ethanol or other addictive drugs is similar to those described above. It is known in the art that certain proteins, *e.g.*, PKA Cα, translocate in response to some cellular stimulus. Using the techniques described herein one of skill in the art can identify those proteins that also translocate in response to cellular exposure to ethanol or other addictive drugs. Other proteins that may translocate upon various cellular stimuli include, but are not limited to, various isozyme-specific receptors for activated PKC (RACKs), such as a δ-PKC specific RACK, RACK 2 (also referred to as β' COP, which is ∈-PKC specific), various scaffolding proteins, such as AKAP 79 (A. Kinase Anchoring Protein) (see, for example, Disatnik et al., *Exp. Cell Res* (1994) 210:287-97; Mochly-Rosen et al., *Science* (1995) 268:247-251; Mochly-Rosen et al., *Biochem Soc Trans.* (1995) 23:596-600; Pawson et al., *Science* (1997) 278:2075-2080). Accordingly, the scope of the invention is not to be limited to the described

embodiments, but rather includes methods that involve detecting any protein localized in different cellular subregions or in a different amount in a given cellular subregion in drug exposed cells (DECs) compared to drug unexposed cells (DUCs), or in cells exposed to other addictive drugs compared to unexposed cells.

The localization of various proteins can be determined in a variety of ways as described below. Generally, cells are examined for evidence of (1) a decrease in the amount of the protein in an origin cellular subregion; (2) an increase in the amount of the protein in a destination cellular subregion (or in an intermediate destination cellular subregion); or (3) a change in the distribution of the protein in the cellular subregions of the cell. The evidence can be direct or indirect. An example of indirect evidence is the detection of a cellular event mediated by the protein including, but not limited to, the cellular events discussed below.

A. Detecting Subcellular Distribution of a Protein

Determination of the localization of the RIα-subunit of PKA, RIIβ-subunit of PKA, Cα-subunit of PKA, δ-PKC, ∈-PKC, or any other ethanol indicative proteins or other proteins which are localized differently in cells exposed to other addictive drugs can be carried out in any of a number of ways. A preferred way is by detection of a colorimetric change, for example, by visual observation. Various methods of visual observation can be used, such as light microscopy, fluorescence microscopy, and confocal microscopy. If desired, an epifluorescence microscope with a CCD camera can be used to measure translocation in the assays described below. This procedure may be automated, for example, by computer-based image recognition. The intracellular distribution of the protein can be determined by staining a cell with a stain specific for the protein. The stain comprises a specific binding substance which binds specifically to the targeted protein. Examples of such a stain include, but are not limited to, antibodies that specifically bind to the protein. A stain specific for, *e.g.*, the ethanol-indicative protein, such as for PKA Cα, PKA RIα, PKA RIIβ, α-PKC, δ-PKC or ∈-PKC can be prepared using known immunocytochemistry techniques. Stains specific for other proteins having cellular locations or quantities that may be correlated with ethanol or other addictive drug exposure may be similarly prepared. Preferably, the stain further comprises a labeling moiety. Suitable antibodies may be prepared using conventional antibody production techniques. The

antibodies may be monoclonal or polyclonal. Antibody fragments, such as, for example Fab fragments, Fv fragments, and the like, are also contemplated. The antibodies may also be obtained from genetically engineered hosts or from conventional sources. Antibodies may be prepared in response to the ethanol indicative protein, *e.g.*, Cα, α-PKC, δ-PKC, ∈-PKC, RIIβ, or RIα, or immunologically reactive fragments thereof. Techniques for antibody production are well known to the person of ordinary skill in the art and examples of such techniques can be found in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1988), Birch and Lennox, Monoclonal Antibodies: Principles and Applications, Wiley-Liss, New York (1995). Examples of suitable antibodies include, but are not limited to, those available from Santa Cruz Antibodies, preferably Lot Nos. B095, B266, G275 and D047 of antibodies that recognize ∈-PKC, Lot Nos. I225 and B226 of antibodies that recognize δ-PKC, and Lot No. I267 of antibodies that recognize PKA-Cα. The labeling moiety will be visibly observable in conventional immunohistochemical detection techniques being, for example, a fluorescent dye such as fluorescein, a chemiluminescense reagent, a radioisotope, a colloidal label, such as colloidal gold or colored latex beads, an enzyme label, or any other known labeling complex. Such stains may be prepared by conventional techniques, for example as described in Manson, Immunochemical Protocols: Methods in Molecular Biology Vol. 10, Humana Press, Totowa, NJ (1992), and Beesley, Immunocytochemistry: A Practical Approach, IRL Press, Oxford, England (1993), the disclosures of which are herein incorporated by reference.

Fusion proteins can also be used to track the localization of a protein. The fusion partner can be detectable directly, such as the green fluorescent protein (GFP), or can be detected indirectly using antibodies specific for the fusion partner or by detecting the enzymatic products of a fusion partner such as ß-galactosidase. Cells which express a fusion protein can be prepared by transfecting a host cell with a polynucleotide encoding the fusion protein. Preferably, the fusion protein is expressed at levels low enough to avoid expression in vast excess of other cellular factors which may be required for subcellular localization of the protein. For example, if a 100-fold molar excess of the fusion protein is expressed relative to a factor required for translocation from the origin subregion to the destination

subregion, translocation upon exposure to, *e.g.*, ethanol, may not be detectable because most of the fusion protein would remain unbound in the origin subregion. This goal can be achieved by not using strong promoters, enhancers or origins of replication giving rise to high copy numbers of plasmids, and by transfecting with smaller amounts of DNA. Preferred fusion proteins include GFP fused to a protein for which its localization is of interest, such as, for example, PKA Cα, PKA RIIβ, δ-PKC, and ∈-PKC. GFP can be fused to either the amino terminus or the carboxy terminus of the protein of interest. A tag, such as a histidine tag, may be included, if desired.

Another preferred way to detect a colorimetric change is to use more than one stain. Preferably, the combination of the stains results in a different color than either stain alone. For example, a cell can be stained with a first stain specific for a particular cellular subregion to be examined and a second stain specific for a particular ethanol indicative protein that migrates to or from that cellular subregion in a cell exposed to ethanol. Examples of such staining systems are known in the art and can be adapted for use in the methods described below. A preferred staining system involves the use of a fluorescence indicator, such as, for example, fluorescein, Cy3, Cy5, Texas Red, rhodamine, and the like. For example, ethanol-treated cells can be stained with antibodies to PKA Cα and secondary antibodies conjugated to fluorescein, which would stain the nuclei green. If the cells are further stained with a red nuclear-specific dye (such as, for example, TOTO-3), the nuclei with PKA Cα will appear yellow instead of red. Other dyes for specific cellular subregions include, but are not limited to, Golgi markers such as mannosidase II and BODIPY TR ceramide (Molecular Probes), nuclear markers such as Neu N, and conjugated antibodies recognizing proteins specific to a particular subregion such as Golgi marker enzymes, histones, and the like.

The particular protein and cellular subregion(s) selected for examination can vary depending on the cell type to be used in a particular method. Cells used in the methods of the invention are of a cell type in which the selected protein is predominantly present in a different amount in a particular cellular subregion of ethanol-exposed cells compared to ethanol-unexposed cells. A difference in intracellular distribution of a particular protein in ethanol-exposed cells can be observed in certain cell types but not others. For example, PKA Cα translocates from

the cytoplasm to the nucleus in cultured neural cells, *e.g.*, the neuroblastoma/glioma cell line NG108-15, exposed to ethanol for 10 minutes or for 12-48 hours, CHO cells exposed to ethanol for 10 minutes, lymphocytes exposed to ethanol for 30 minutes or for 12-48 hours, and brain Purkinje cells exposed to ethanol for 72 hours, but not in neutrophils. Table 1 provides a list of the observed intracellular distribution of some ethanol indicative proteins in some cell types. One of skill in the art can readily determine the intracellular distribution of other ethanol indicative proteins by staining ethanol-exposed and ethanol-unexposed cells of various cell types with stains (*e.g.*, antibodies) specific for such proteins. Other useful ethanol indicative proteins are likely to be proteins that are known to translocate in response to some cellular stimulus, such as, for example, activation by cAMP, cGMP, phorbol myristate acetate (PMA), phosphorylation, or inactivation by dephosphorylation. Most preferred are kinases and phosphatases, such as, for example, calcium/calmodulin dependent kinases, and protein phosphatases 1, 2A, 2B, and MAP kinase. Other proteins that translocate include calcium/calmodulin and transcription factors.

Table 1

Localization of Ethanol – Indicative Proteins

Cell Type	Ethanol-Indicative Protein	Cellular Subregion in which the Protein is Predominantly Present	
		EUC (Origin)	EEC (Destination)
NG108	PKA Cα	Golgi	Nucleus & cytoplasm (10' exposure) Nucleus (12-48hr exposure)
	δ-PKC	Golgi	Perinucleus and nucleus
	∈-PKC	Perinucleus	Cytoplasm
	RIIβ	Golgi	Nucleus
	RACK 2 (β'COP)	Perinucleus	Cytoplasm
	Protein Phosphatase I and IIB	Nucleus	Cytoplasm
Lymphocyte	PKA Cα	Cytoplasm	Nucleus
	PKA RIα	Cytoplasm	Nucleus
	PKA RIIβ	Cytoplasm	Cytoplasm
	δ-PKC	Cytoplasm	Cytoplasm
	∈-PKC	Cytoplasm	Cytoplasm
Neutrophil	PKA Cα	Cytoplasm	Cytoplasm
	PKA RIα	Cytoplasm	Nucleus
CHO	PKA Cα	Golgi	Cytoplasm (10' exposure) Nucleus (12-48hr exposure)
	δ-PKC	Golgi	Perinucleus and nucleus
	∈-PKC	Perinucleus	Cytoplasm (10' exposure)
	RACK 2 (β'COP)	Perinucleus	Cytoplasm (10' exposure)

Cell Type	Ethanol-Indicative Protein	Cellular Subregion in which the Protein is Predominantly Present	
		EUC (Origin)	EEC (Destination)
Purkinje cells in brain section	PKA Cα	Cytoplasm	Not detectable (72 hr exposure)
	∈-PKC	Cytoplasm	Not detectable (72 hr exposure)

*Unless otherwise indicated, the protein is predominantly present in the destination cellular subregion in cells exposed to ethanol for either 10 minutes or for 12-48 hours.

B. Detecting Cellular Events Induced by an Ethanol-Indicative Protein

A change in the cellular localization of a protein in a cell exposed to ethanol or other addictive drugs can trigger certain cellular events that can be detected. Examples of such events include phosphorylation of substrate proteins, gene regulation, changes in cytoskeletal structure, release of synaptic vesicles and the like. Such cellular events can be examined in a variety of ways as discussed in greater detail below.

1. Phosphorylation

Another aspect of the invention is to provide methods for detecting the effects of ethanol or other addictive drugs on cells by measuring the phosphorylation of proteins that are differentially phosphorylated in the presence and absence of ethanol. As previously discussed, exposure of cells to ethanol results in the translocation of Cα to the nucleus, where the Cα catalytic subunit may phosphorylate serine and/or threonine targets in a set of proteins that differs from the set of proteins available for phosphorylation in the cytoplasm, plasma membrane or Golgi. One such protein is CREB.

The identity of other proteins that are differentially phosphorylated in response to ethanol exposure may readily be determined using conventional assay techniques known to the person of skill in the art. For example, radioactively labeled phosphate may be added to cultured cells grown in both the presence and absence of ethanol. Proteins from the labeled cells may then be extracted and separated on a one or two dimensional gel system. Isolated phosphorylated proteins may then be visualized by autoradiography and related techniques. After separation and visualization, changes in the level of phosphorylation of different proteins may be determined by comparing the results obtained from cells exposed to ethanol with the results obtained from cells

not exposed to ethanol. Preferably, proteins of interest are immunoprecipitated. Proteins that are differentially phosphorylated by an ethanol indicative protein in response to ethanol may be identified by amino terminus amino acid residue sequencing.

A more sensitive detection method involves the use of phosphoantibodies, for example, antibodies that recognize phosphorylated forms of specific proteins, or antibodies that recognize a phosphorylated amino acid residue, such as phosphothreonine or phosphoserine antibodies. Another useful detection method is back-phosphorylation, which is safer than direct phosphorylation assays but less sensitive. Cell extracts are incubated with radiolabeled ATP and Mg^{++} and subjected to gel electrophoresis. Since ethanol alters phosphorylation, a different amount of radiolabeled phosphate will be incorporated into individual proteins of cells exposed to ethanol than in cells which have not been so exposed, resulting in a different pattern of bands on a gel.

Proteins that are differentially phosphorylated in response to cellular ethanol exposure may be used in assays for the exposure of cells to ethanol. Furthermore, these differentially phosphorylated proteins may be used as the targets when screening for compounds that modulate the cellular effects of ethanol. Such assays include assays involving the steps of measuring the phosphorylation of differentially phosphorylated proteins. Compounds could be screened by measuring their effects on phosphorylation of these differentially phosphorylated proteins.

Phosphorylation of such proteins by an ethanol indicative protein in response to cellular exposure to ethanol can be determined in a variety of ways known in the art, such as, for example, by using phospho-specific antibodies specific for various proteins in the signal transduction pathway. Such antibodies are available commercially (*e.g.*, New England Biolabs (NEB), Inc., 32 Tozer Road, Beverly, MA 01915). Examples of suitable phospho-specific antibodies include, but are not limited to, anti-phospho CREB antibodies, such as anti-phospho CREB (Ser 133) polyclonal antibody (NEB #9192).

2. Gene Expression

Some proteins which are localized differently in cells exposed to ethanol or other addictive drugs can affect gene regulation, either directly or indirectly. For

example, upon migration away from the Golgi, PKA Cα causes the phosphorylation and activation of CREB, which regulates the expression of genes having regulatory regions which contain a cAMP response element (CRE). CRE-regulated genes include, but are not limited to, genes encoding receptors for γ-aminobutyric acid (GABA receptors) and receptors for N-methyl-D-aspartate (NMDA receptors), which are thought to underlie some behavioral patterns observed in alcoholics. Other factors involved in transcription may also be activated upon migration of PKA Cα. For example, it is thought that, upon binding to phosphorylated CREB, CREB binding protein (CBP) sets the transcription machinery in motion. Other proteins that migrate to the nucleus include δ-PKC, PKA RIIβ, PKA RIα, MAP kinase and calcium/calmodulin dependent kinases, such as CaM kinases I, II and IV. For purposes of the methods described below, the gene is preferably regulated by a factor phosphorylated or dephosphorylated (whether directly or indirectly) by the protein. Phosphorylation or dephosphorylation of the factor alters the expression of the gene. For example, expression can be upregulated by certain factors when such factors are phosphorylated. Some factors, when dephosphorylated no longer increase expression. For example, CREM (CREB modulator) and CaM kinase negatively regulate gene expression by inhibiting CREB mediated expression.

Preferably, the regulated gene is a reporter gene, the expression of which is preferably controlled by CRE. Control of expression by CRE can be enhanced by increasing the number of binding sites for phosphorylated CREB and/or decreasing the number of CRE-independent transcription control sequences present in the vicinity of the reporter gene. Examples of reporter genes, include, but are not limited to chloramphenicol acetyl transferase (CAT) (Alton et al., *Nature* (1979) 282:864-869), beta-galactosidase, firefly luciferase (deWet et al., *Mol. Cell. Biol.* (1987) 7:725-737), bacterial luciferase (Engebrecht et al., *Proc. Natl. Acad. Sci. USA* (1984) 1:4154-4158; Baldwin et al., *Biochemistry* (1984) 23:3663-3667, alkaline phosphatase (Toh et al., *J. Biochem.* (1989) 182:231-238; Hall et al., *J. Mol. Appl. Gen.* (1983) 2:101, and green fluorescent protein (GFP) (Meyer et al., *Diabetes* (1998) 47(12):1974-1977), a GFP-luciferase fusion protein (Day et al. *Biotechniques* 1998 25(5):848-850, 852-854, 856), and other genes encoding a detectable gene product. Detection of gene expression can be achieved in a variety of ways depending on the reporter gene used. For example, a fluorescence or chemiluminescence detection

system can be used to detect expression of luciferase and GFP. A CREB-dependent GFP construct is preferred. Alternatively, an antibody that recognizes the gene product encoded by a reporter gene can be used to detect expression of many reporter genes as well as many endogenous CRE-regulated genes. Visual observation of a colorimetric change can be used to detect expression of beta-galactosidase or alkaline phosphatase. A reporter gene can be inserted into the cells by various techniques known in the art and described herein. Transient expression is preferred. However, the reporter gene can be present on a vector that is stably integrated into the genome of the cells.

The expression of genes in response to the presence of an ethanol-indicative protein in the nucleus can be monitored by any of a number of ways known in the art and described herein, such as, for example, by Northern analysis, polymerase chain reaction (PCR), Western analysis, radioimmunoassays (RIA), enzyme linked immunoassays (ELISA or EIA), fluorescence activated cell sorting (FACS) analysis, enzyme-substrate assays such as chloramphenicol transferase (CAT) assays, and the like. Preferably, expression of such genes in response to cellular exposure to an addictive drug is determined by detecting a signal at least about 1.5 times that of control cells which have not been exposed to the addictive drug, preferably greater than about 2X.

C. Methods for Determining Exposure to an Addictive Drug

In one aspect, the present invention provides methods for detecting prior and chronic exposure of a sample to ethanol or other addictive drugs. Such methods are useful for a variety of purposes, such as for determining whether an individual has been actively consuming alcohol or other addictive drugs recently or over an extended period of time. Such determinations can be valuable for monitoring compliance with a rehabilitation program for alcoholism or other addictive disease, or for an extension of a drug testing program in the workplace. In addition, a diagnostic test is useful in evaluating all patients undergoing medical care as ambulatory or hospitalized patients. For example, this could be used in a medical screening clinic since 7-10% of the population are alcoholics. Among hospitalized patients, it is estimated that 25-65% have an alcohol-related diagnosis. Some methods of the invention can be used to

distinguish between chronic and current consumption in an individual, usually where there is an undetectable level of the addictive drug in the sample.

The methods for detection of exposure to, *e.g.*, ethanol, fundamentally involve detecting the presence or absence of an ethanol indicative protein in a particular cellular subregion of a test cell in the sample, the test cell being of a cell type in which the protein is present in the cellular subregion in a detectably different amount in an EEC than in an EUC. Usually, the protein is distributed differently between an EEC versus an EUC. This amount is compared to the amount of the protein present in the cellular subregion in a control cell of a control sample which has not been exposed to ethanol. The detection of a difference relative to the control is indicative of ethanol exposure. An alternate control is one comprising a cell that has been exposed to ethanol. The detection of a similar amount of the protein relative to the alternate control is indicative of ethanol exposure. These methods are described below and involve detection methods described in greater detail above.

1. Methods for Determining Cellular Exposure to an Addictive Drug

In one aspect, the present invention provides a method for determining prior exposure to ethanol or other addictive drugs of a sample containing at least one cell, comprising identifying a protein having a localization that is substantially affected by exposure to the addictive drug, and determining the distribution of the protein within the cell, thereby producing an indication of the exposure of the cell to the addictive drug. In one specific embodiment, the protein is PKA Cα or PKA RIα. In another specific embodiment, the protein is the δ- or ∈-isozyme of the protein kinase C (PKC). Preferably, the sample contains a plurality of cells, and the determination is carried out for several of the plurality of cells. Preferably, the cells analyzed are derived from blood samples, *e.g.*, lymphocytes, granulocytes, etc. The cell type, protein and drug chosen should be compatible in that the cell type should be one in which the protein to be analyzed translocates in response to the drug of interest. For instance, if one desires to determine cellular exposure to ethanol by determining the localization of δ-PKC or ε-PKC, fibroblasts would be a preferred cell type and human lymphocytes would not be advisable since neither δ-PKC nor ε-PKC translocate in these cells following ethanol exposure. Similarly, it would not be advisable to use

human blood samples to determine exposure to cocaine since these cells lack norepinephrine transporters. In contrast, human blood cells do contain opioid, cannabinoid and dopamine receptors and can be used to determine exposure to addictive drugs that act through such receptors. Human blood cells may contain nicotinic receptors which would allow them to be used to determine exposure to nicotine.

The step of identifying preferably comprises staining the cell with a staining complex having specific binding affinity for the protein. The step of determining the location of the protein preferably includes imaging or observing the cell using conventional imaging techniques, *e.g.*, a microscope. Nuclear accumulation of a protein, such as an ethanol indicative protein, *e.g.*, the Cα of PKA, δ-PKC or ∈-PKC can also be assessed by observing Cα-, RIα-, RIIβ-, δ-PKC-, or ∈-PKC-induced cellular events. For example, it may be possible to observe the chronic activation of CREB transcription factor and other nuclear substrates in response to Cα-activation. Preferably chronic activation of such nuclear substrates is detected by using phospho antibodies that specifically bind to the activated substrates discussed above, or by monitoring the expression of endogenous genes regulated by the nuclear substrates, such as, for example, NMDA receptor, enkephalin, glucose transporters, phosphodiesterase, muscarinic (M3) acetylcholine receptor, β_3-adrenergic receptor, α_1 adrenergic receptor, and the like. Such expression studies should be carried out in cells in which the encoded proteins are normally present. For example, it would not be advisable to use blood cells to assess expression changes of the NMDA receptor or enkephalin because these proteins are not normally present in blood cells. While both α and β adrenergic receptors are present on blood cells, their types would need to be confirmed before using such cells to monitor expression of genes regulated by the β_3-adrenergic receptor or α_1 adrenergic receptor. Some of these proteins may also be regulated by other signaling cascades that are in turn regulated by ethanol. Therefore, although these proteins have CREs and the expression of these proteins would be increased by just increasing the cAMP levels, their expression could by altered by ethanol in a PKA-independent manner as well. Suitable detection methods are described in greater detail above. Methods for determining exposure to ethanol are described in detail below and can readily be applied to determining exposure to other addictive drugs as well.

The invention provides methods for determining exposure of cells to ethanol comprising the step of applying a stain using specific affinity for the ethanol indicative protein, *e.g.*, the Cα-subunit of PKA, or the δ- or ∈-PKC isozyme to the sample so as to identify the region or regions of the cell that contain Cα, δ-PKC, or ∈-PKC, respectively. After the regions of the cell containing PKA or PKC have been identified, the cell or cells are classified as to the distribution of the stain within the cells, wherein localization of Cα stain in the cell nucleus, δ-PKC in the perinucleus and the nucleus, and ∈-PKC stain in the cytoplasm is indicative of prior exposure of the cell to ethanol. Cells that contain significant, *i.e.*, greater than control cells, detectable amounts of stain for Cα of PKA in the nucleus, the δ-subunit of PKC in the perinucleus and nucleus, or ∈-PKC in the cytoplasm, respectively, are indicative as being exposed to ethanol.

Advantageously, the determination of cellular localization of the ethanol indicative protein, *e.g.*, Cα, δ-PKC or ∈-PKC, comprises identifying first and second regions within each cell, and classifying cells as a first type if the protein is predominately present in a first region, and as a second type if the protein is predominately present in the second region. The number of cells of the first type may be compared to the number of cells of the second type for a determined number of cells within a sample. A number dependent on the proportion of cells of the first and second types, usually the ratio or percentage, may be correlated with a control derived from reference data to obtain a qualitative determination of whether exposure to ethanol of the sample has exceeded a certain threshold, or to obtain a semi-quantitative determination of the exposure to ethanol of the sample. In the case of Cα, the first region will preferably be the nucleus of the cell, and the second region will preferably be the perinuclear Golgi apparatus. In the case of δ-PKC, the first region will preferably be the perinucleus and the nucleus of the cell, and the second region will preferably be the perinuclear Golgi apparatus. In the case of ∈-PKC, the first region will preferably be the cytoplasm of the cell, and the second region will preferably be the perinucleus and the nucleus of the cell. The control will be of the type of cells being examined, and usually fewer than about 25-35% of the cells display localization in the first region, preferably fewer than about 10-15%, with less than about 1-5% being most preferred. Stated another way, if more than about

25-35 % of the cells display localization in the first region, the sample will be positive for localization in the first region.

It will be readily appreciated by one of skill in the art that, depending on the cell type examined, a sample can be classified as positive for ethanol exposure based on lower or higher percentages of cells displaying localization in the first region. One factor affecting the observed percentages is the half-life of a particular cell type. More cells of an immortalized cell line, such as a neuroblastoma cell line, CHO cell line, and the like, are expected to display altered localization than primary cells such as lymphocytes, neutrophils, and the like. Furthermore, the half-life of lymphocytes is about two weeks, whereas the half-life of neutrophils is about eight hours. Thus, the absolute number of cells displaying localization changes can vary depending on the amount of time that has passed since the last exposure to ethanol. However, a detectably greater number of cells exposed to ethanol will display localization in the first region, preferably a statistically significant greater number, more preferably at least about 10% more cells, even more preferably at least about 25% more cells, with more than about 40% cells being most preferred.

The classification step includes the step of identifying the cellular location of the stain and hence the location of the ethanol indicative protein, *e.g.*, the Cα-subunit of PKA, δ-PKC or ∈-PKC or any other proteins with comparable localization behavior. The precise means of identifying the cellular location of the ethanol-indicative protein, *e.g.*, Cα, δ-PKC or ∈-PKC will vary with label used in the stain. Generally, a variety of methods may be used for each type of label selected. For example, a radioisotope label may be detected through film (autoradiography), Charge Coupled Devices (CCDs) and the like.

When analyzing the results of the subject assays in which a sample containing a plurality of cells is stained with a specific stain for an ethanol indicative protein, *e.g.*, Cα, δ-PKC or ∈-PKC, the percentage of cells that show altered location of the ethanol indicative protein must be considered. In some instances, not every ethanol exposed cell in a sample will show altered location of the ethanol indicative protein. However, significantly more cells with altered location of the ethanol indicative protein will be found in multiple cell containing samples that have been exposed to ethanol as opposed to control samples. Furthermore, the percentage of cells showing altered location of the ethanol indicative protein, *i.e.*, translocation to the nucleus in

the case of Cα, translocation to the perinucleus and the nucleus in the case of δ-PKC, or translocation to the cytoplasm in the case of ∈-PKC, is expected to increase with increasing duration of exposure to ethanol and with increasing amount of ethanol to which the cells are exposed. Statistical analysis may be used to develop quantitative correlations between the percentage of cells in a sample sharing altered location of the ethanol indicative protein and the amount of exposure. Other factors to consider when making such correlations include the age and condition of the source of the cell sample, the particular cell type being analyzed, and the like. The sample may be taken from a live subject, for example a human whose ethanol consumption is to be determined. Alternatively, cells cultured *in vitro* may be used in those embodiments of the invention that are directed to the monitoring of ethanol in subjects, *e.g.*, screening for therapeutic agents. Where the sample is taken from a live subject, the sample is preferably a blood-sample, containing nucleated cells, such as granulocytes and lymphocytes.

2. Methods for Determining Chronic Cellular Exposure to an Addictive Drug

The invention also provides methods of determining a subject's chronic cellular exposure to ethanol or other addictive drugs. Such methods relate to the discovery in cells obtained from a subject that, unlike cells that have not been chronically exposed to ethanol, cells which have been chronically exposed to ethanol continue to exhibit the effects of ethanol on cellular localization of some ethanol indicative proteins regardless of the presence of ethanol in the sample before it is prepared for analysis (see Example 4).

The methods involve determining cellular exposure to an addictive drug of a sample by detecting the amount of an ethanol indicative protein in a particular cellular subregion(s) of at least one test cell in the test sample as described above, and comparing the results to those obtained with a control sample which has not been exposed to ethanol. Many alcoholics are able to achieve blood ethanol concentrations approaching or exceeding those required *in vitro* for altered localization. Once achieved, the state of altered localization is maintained for a time even through the blood level of ethanol drops below the threshold level. Thus, when comparing samples from an alcoholic and a nonalcoholic having a blood ethanol level of less

than about 200 mM, preferably less than about 25mM, the alcoholic, but not the nonalcoholic, is expected to exhibit altered localization.

3. Methods for Determining Consumption of an Addictive Drug

The consumption of an addictive drug by a subject, particularly a human, could in principle be determined by assaying the cells obtained from the subject. Cells for analysis in the subject assays for addictive drug exposure may come from a variety of locations within the body. Cell containing samples may be obtained from organs or non-organ tissue. Preferably, cell containing samples are obtained from easily removed tissues such as blood and skin. Because of the transient and reversible effects of ethanol on ethanol indicative proteins, *e.g.*, Cα, δ-PKC, ∈-PKC, it is important that cellular samples be fixed in, *e.g.*, methanol, acetone, formaldehyde or paraformaldehyde prior to analysis, or be analyzed with the assays of the invention as soon as possible after the sample is removed from a subject for analysis. Localization of ethanol indicative proteins or addictive drug indicative proteins such as the PKA Cα or PKA RIα (or other proteins localized differently in cells exposed to addictive drugs compared to unexposed cells) in granulocytes and/or lymphocytes can be investigated, provided that the receptor for the addictive drug of interest is expressed in such cells. Both of these cell types can be conveniently obtained from blood samples. For example, to determine the effect of ethanol consumption in a particular individual, a comparison can be made between the proportion of cells having, *e.g.*, predominately nuclear localization of Cα to that obtained from a reference sample. To monitor progress of the individual over time, a number of samples can be taken, and variations in the localization of staining can be monitored. The technique can be used to determine the effects of a treatment on a live subject, by monitoring changes in the subject when provided with the treatment. For example, since the half-life of lymphocytes is about two weeks, PKA Cα should be detected in the nucleus of only about half as many lymphocytes in a sample taken two weeks after a first sampling if there has been no further exposure to ethanol.

4. Methods for Determining Chronic Consumption of an Addictive Drug

The methods described above for determining chronic cellular exposure to an addictive drug can be applied to determining whether a mammal has been chronically consuming an addictive drug. For example, a mammal, such as a human, that has been chronically consuming ethanol is frequently an alcoholic.

Several methods are used in the art to characterize an individual as having a substance use disorder, *e.g.*, being an alcoholic or other addict. A person suffering from such disorders suffers from substance dependence and/or substance abuse, which are defined in the art as a maladaptive use of the substance, leading to clinically significant impairment or distress, as manifested by certain conditions, occurring at any time in the same 12-month period. Substance dependence is diagnosed by three or more of the following conditions: (1) tolerance; (2) withdrawal; (3) often taking the substance in larger amounts or over a longer period than was intended; (4) persistent desire or unsuccessful efforts to cut down or control substance use; (5) a great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects; (6) important social, occupational, or recreational activities are given up or reduced because of substance use; (7) the substance use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance. Tolerance is defined by either a need for markedly increased amounts of the substance to achieve intoxication or desired effect, or a markedly diminished effect with continued use of the same amount of the substance. Withdrawal is manifested by the characteristic withdrawal syndrome for the substance, or the same or closely related substance is taken to relieve or avoid withdrawal symptoms. Alcohol withdrawal, for example, is manifested by two or more symptoms of autonomic hyperactivity (*e.g.*, sweating or pulse rate greater than 100); increased hand tremor; insomnia; nausea or vomiting; transient visual, tactile, or auditory hallucinations or illusions; psychomotor agitation; anxiety; and grand mal seizures. Substance abuse is diagnosed by one or more of the following conditions: (1) recurrent substance use resulting in a failure to fulfill major role obligations at work, school, or home; (2) recurrent substance use in situations in which it is physically hazardous; (3) recurrent substance-related legal problems; or (4) continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance.

The most preferred method of characterizing an individual as having a substance use disorder is a psychological evaluation, for example, an evaluation using an ICD-10 questionnaire such as the one shown in Table 3. Another preferred method is by determining the level of consumption of ethanol by the individual (Watson et al., *J. Clin Psych* (1995) 51:676-684; Dawson et al, *Addiction* (1994) 89:345-350; Hasin et al., *Alcoholism: Clin Exp Res* (1998) 22-580-584; Dawson, *J. Studies on Alcohol* (1998) 59:191-197; Tivis et al., *Alcoholism: Clin Exp Res* (1999) 22:902-907). For example, a human who consumes about 168 drinks in 4 weeks (about 90 g/day) or who has an average daily consumption of about 6 drinks/day can be characterized as an alcoholic. A preferred method comprises obtaining a cellular test sample from the mammal, detecting the amount of an ethanol indicative protein in a particular cellular subregion(s) of at least one test cell in the test sample as described above, and comparing the results to those obtained with a control sample which has not been exposed to ethanol. In one embodiment, the blood alcohol level of this mammal is undetectable by conventional methods.

Preferably, if the sample is a blood sample, the ethanol indicative protein examined is PKA Cα or PKA RIα. The half-life of neutrophils is about 8 hours, whereas the half-life of lymphocytes is about 2 weeks. Thus, as the length of time from the last exposure to ethanol increases, the number of cells exhibiting ethanol-mediated cellular localization effects decreases. A preferred assay examines the amount of PKA Cα present in the nucleus of lymphocytes.

D. Methods for Identifying Substances that Alter or Mimic the Effects of an Addictive Drug

A second aspect of the invention is to provide screening methods for substances that can alter or mimic the effects of ethanol or other addictive drugs on the cellular localization of a protein. Substances that alter or mimic the effects of ethanol on cellular localization of proteins would be useful for a variety of purposes. One such purpose is as a research tool to study the role of cellular localization of a protein in various aspects of alcoholism or other addictive disease, such as behavior, dependency, etc. Another purpose is to develop drugs for the treatment of alcoholism or other addictions or the prevention of certain manifestations of the diseases. For example, an inhibitor may permit consumption of alcohol without experiencing some

of the negative effects of such consumption. An enhancer or a mimic might substitute for ethanol without the detrimental effects, somewhat analogous to the effects of methadone for a heroin addict.

Substances that can be screened for such activity include elements and compounds. Examples of test compounds include, but are not limited to, inorganic compounds, small organic compounds prepared by combinatorial chemistry, known pharmaceutical compounds, combinatorial peptide libraries, compounds or peptides produced by rational drug design, nucleic acid-based compounds, such as, for example, antisense compounds, antibodies or antibody fragments, polysaccharides, and the like. Compounds known to affect pathways believed to be involved in translocation of ethanol indicative proteins can be tested for their ability to alter or mimic the effects of ethanol. Some preferred classes of compounds include agonists and antagonists of adenosine A1 receptors, adenosine A2 receptors, dopamine D2 receptors, δ-opiate receptors, cannabinoid receptors, PKA, phospholipase C (PLC) and PKC, as well as inhibitors and activators of adenylyl cyclase, of G_i, G_o and G_z mediated functions and of translocation of PKC (for example, those described in U.S. Patent Nos. 5,519,003 and 5,783,405) or PKA. Also preferred are compounds which are structurally related to those described above.

The detection methods described above can be used in such screening methods to determine whether the substances tested have the desired activity. Such methods can include assays in which PKA RIα, PKA RIIβ, PKA Cα, α-PKC, δ-PKC or ∈-PKC cellular localization is determined and/or measured.

The sample can be a tissue sample, such as neural tissue or skin tissue, and is preferably a cell culture. Cells for use in the subject screening assays for compounds that modulate the effects of ethanol are of a cell type in which an ethanol indicative protein is present in a particular cellular subregion in a detectably different amount in an ethanol-exposed cell than in an ethanol-unexposed cell. The cells may be primary cells derived directly from a subject or may be cells from a cell line. Primary cells are cells which can be propagated for only a finite number of cell divisions, *i.e.*, cells which are not immortal. Some preferred primary cells include neuronal cells, such as, for example, glial cells and Purkinje cells and blood cells such as lymphocytes and granulocytes. Cells for use in the assays of the invention may be obtained from cells cultured *in vitro*. Preferably, the cells used in the assay are from cell line cells.

Immortalized cell lines are preferred for use in the subject screening assays in part because they provide more consistency between assays. Examples of immortalized cell lines include tumor cell lines or cell lines derived from fusion of a non-tumor cell with a tumor cell. More preferably the cells are of a cell type derived from brain or neural tissue, such as a neuroblastoma, particularly NG108-15 neuroblastoma X glioma cells, are from a fibroblast cell line, or are from cell lines derived from lymphocytes or granulocytes, such as, for example, HL-60 cells, Jurkat cells, S49 mouse lymphoma cells, and a Friend human leukemia cell line. Most preferred are cells that can be easily cultured in a defined media, preferably a serum-free media (in part because serum contains factors which can interfere with the assay), and are sufficiently adherent so as to remain attached to a surface during wash steps in the assay, such as, for example, NG108-15 cells or Chinese hamster ovary (CHO) cells which have been adapted to grow well for at least about 48 hours in serum-free media or serum-depleted media, respectively.

A preferred assay for detecting the cellular localization of a particular ethanol-indicative protein in the screening methods of the invention comprises detecting a colorimetric change, as described above. Where such assays involve staining or labeling of the cells with a stain or label specific for the protein the test cells and/or control cells can be stained or labeled before exposure to ethanol and/or the substance if the staining or labeling would not interfere with cellular localization in response to exposure to ethanol and/or the substance. Usually the cells are stained or labeled after exposure to ethanol and/or the substance. In one embodiment, the detection assay comprises observing the distribution of a stain specific for the protein by observing the accumulation of stain in one cellular subregion compared to a second cellular subregion. Alternatively, cells can be examined for the presence of the stain in a particular cellular subregion without regard for the presence of the stain in a different cellular subregion.

In yet another embodiment, the localization of a protein of interest can be observed in cells transfected with a polynucleotide encoding the protein of interest fused to a polynucleotide encoding a marker polypeptide which permits detection of the fusion protein in a particular cellular subregion. Preferably, the cells are stably transfected. For example, visual inspection of, *e.g.*, CHO cells expressing a PKA Cα - GFP fusion protein would reveal fluorescence in the Golgi apparatus of cells not

exposed to ethanol and fluorescence in the nucleus of cells exposed to ethanol for 12-48 hours. One could examine these cells for changes in this pattern when treated with a test substance.

In another embodiment, the cells can be stained or labeled with a first substance specific for the target cellular subregion and a second substance specific for a particular ethanol indicative protein. Preferably, the first and second substances are substances that produce a colorimetric change when combined, such as, for example, substances used in fluorescence resonance energy transfer (FRET).

Another preferred detection assay comprises detecting the expression of a gene regulated by cellular events mediated by a particular protein, such as an ethanol indicative protein, as described above. Particularly preferred is an assay detecting the expression of a reporter gene regulated by CREB, such as a heterologous luciferase gene operably linked to a CRE recognition sequence as described in greater detail in Example 6. Another preferred reporter system is one in which cells are co-transfected with a GAL4-CREB fusion construct and with a second construct containing the DNA binding site for GAL4 fused to luciferase as a reporter gene, such as the PathDetect™ trans-acting system (see Example 6). Suitable cells for use in a reporter system include neuroblastoma cells, such as NG108-15, Chinese hamster ovary (CHO) cells, HEK 293 cells, other fibroblast cells, primary cultures of neuronal cells, such as glia or neurons, other primary cells such as lymphocytes, and other cells in which migration of an ethanol indicative protein to the nucleus either promotes or inhibits gene expression, with NG108-15 and CHO cells being most preferred. Other suitable reporter genes are described in greater detail above.

1. Methods for Screening for Substances that Alter the Effects of an Addictive Drug on Cellular Localization

In a preferred embodiment, the invention provides a method for identifying a substance that alters the effects of ethanol or other addictive drugs on the cellular localization of a protein. Typically, the method comprises exposing a test sample comprising at least one test cell to an addictive drug or a receptor agonist exerting the same effects on the localization of a protein as an addictive drug, exposing the test cell to the substance, and detecting the presence or absence of a protein in a particular cellular subregion. This amount can be compared to the amount of the protein present in the corresponding cellular subregion of a control cell of a control sample which has

been exposed to the addictive drug but not to the substance. If a different amount of the protein is detected in the cellular subregion in a test cell exposed to the substance and the addictive drug compared to a control cell exposed to the addictive drug alone, the substance is considered to have altered the effects of the addictive drug on the cellular localization of the protein.

In part because of the experimental evidence discussed in Example 7, the inventors believe that addictive drugs may involve the same or similar signal transduction pathways. In addition, as shown in Table 6, agonists of several classes of receptors exert the same effects on the subcellular localization of some proteins as is observed with ethanol. Thus, receptor agonists suitable for the screening methods of the invention include, but are not limited to, adenosine receptor agonists, dopamine receptor agonists, δ-opiate receptor agonists, and cannabinoid receptor agonists.

The test sample can be exposed sequentially or simultaneously to the addictive drug or agonist and the substance. When exposed sequentially, the sample is preferably exposed to the substance before the addictive drug or agonist. Data from the control cell can be obtained during the same experimental period as the test cell. Alternatively, the protein can be detected in the control cell in advance of experiments testing a specific substance or during the course of experiments testing a different substance. Those data can provide a reference standard against which to compare data obtained from experiments testing the effects of various substances.

In a preferred embodiment, the test sample comprises a plurality of test cells and the control sample comprises a plurality of control cells. Where the sample comprises a plurality of cells, the method can comprise detecting a minimum percentage of the test cells having a detectably different amount of the protein in the cellular subregion examined. The percentage is a detectably different percentage from that of control cells having a detectably different amount of the protein in the cellular subregion. Preferably, the substance is considered to have altered the effects of ethanol on the cellular localization if the difference in amount is detected in at least a statistically significant greater number of test cells compared to control cells, preferably greater than 5%, more preferably greater than 15%, even more preferably greater than 25%, with greater than 35%, up to 100%, being most preferred, depending on a variety of factors, including, but not limited to, the detection method used.

The test cells can be exposed to the addictive drug and the test substance either simultaneously or separately. When exposed separately to the addictive drug and the substance, the test cells are preferably exposed to the substance before the drug.

2. Examining the Destination Cellular Subregion

Depending on the particular ethanol indicative protein, cellular subregion, and cell type studied, the protein can be present in the cellular subregion in a detectably greater amount in, *e.g.*, EECs than in EUCs. This can occur, for example, when the protein translocates to the cellular subregion in cells exposed to ethanol. Usually, the protein has translocated from an origin cellular subregion to a destination cellular subregion such that the protein is present in a detectably greater amount in the destination cellular subregion than in the origin cellular subregion in EECs, as shown in the Examples.

The substance can be considered an inhibitor of the effects of ethanol on the cellular localization of the protein if the amount of protein in the destination cellular subregion of the test cells is less than that of control cells which have been exposed to ethanol but not to the substance. Conversely, the substance can be considered an enhancer of the effects of ethanol on the cellular localization of the protein if the amount of protein in the destination cellular subregion of the test cells is greater than that of the control cells.

A substance can also be classified as an inhibitor or an enhancer depending on the number of test cells in which the protein is present in the first cellular subregion. For example, a substance can be classified as an inhibitor if the protein is present in the destination cellular subregion in a detectably lesser number of the test cells than in the control cells. Conversely, the substance can be classified as an enhancer if the protein is present in the destination cellular subregion in a detectably greater number of the test cells than in the control cells.

3. Examining the Origin Cellular Subregion

Depending on the particular ethanol indicative protein, cellular subregion, and cell type studied, the protein can be present in the cellular subregion in a detectably lesser amount in, *e.g.*, EECs than in EUCs. This can occur, for example, when the protein is no longer detectable in the cellular subregion in cells exposed to ethanol. In

some instances, the protein translocates to a second detectable cellular subregion (a destination cellular subregion). In other instances, the protein can be detected in EUCs, but is undetectable by some detection methods in any other cellular subregion in EECs. The protein may have been degraded, may have assumed an altered conformation, or may have translocated to a region of the cell where it is not detectable by the detection method used. This is the case, for example, for PKA Cα and ∈-PKC in Purkinje cells of cerebellum tissue sections. These proteins are detectable in the cytoplasm in EUCs, but are not detectable at all by immunofluorescent microscopy in EECs.

The substance can be considered an inhibitor of the effects of ethanol on the cellular localization of the protein if the amount of protein in the origin cellular subregion of the test cells is greater than that of control cells which have been exposed to ethanol but not to the substance. Conversely, the substance can be considered an enhancer of the effects of ethanol on the cellular localization of the protein if the amount of protein in the origin cellular subregion of the test cells is less than that of the control cells.

A substance can also be classified as an inhibitor or an enhancer depending on the number of test cells in which the protein is present in the origin cellular subregion. For example, a substance can be classified as an inhibitor if the protein is present in the origin cellular subregion in a detectably greater number of the test cells than in the control cells. Conversely, the substance can be classified as an enhancer if the protein is present in the origin cellular subregion in a detectably lesser number of the test cells than in the control cells.

4. Methods for Screening for Substances that Mimic the Effects of an Addictive Drug on Cellular Localization

The invention also provides a method for identifying a substance that mimics the effects of an addictive drug on the cellular localization of a protein. Typically, the method comprises exposing a sample comprising at least one test cell to the substance but not to the addictive drug, and detecting the amount of the protein in a particular cellular subregion. This amount can be compared to the amount of the protein present in the corresponding cellular subregion of a control cell which has been exposed to the addictive drug but not to the substance. If a similar amount or distribution of the

protein is detected in the cellular subregion in a test cell exposed to the substance compared to a control cell, the substance is considered to have mimicked the effects of the addictive drug. Data from the control cell can be obtained as discussed above in the previous section.

In a preferred embodiment, the sample comprises a plurality of test cells and a plurality of control cells. Where the sample comprises a plurality of cells, the method comprises detecting a percentage of the test cells having an amount of the protein in a particular cellular subregion being examined or a distribution of the protein similar to that of EECs. Detection of a minimum percentage can be indicative of the substance having mimicked the effects of, *e.g.*, ethanol on the cellular localization of the protein. The minimum percentage is preferably a detectably greater percentage than a percentage of cells of a second control which have not been exposed to either ethanol or the substance.

As discussed above in the previous section, the method can involve examining either the cellular subregion in which more of the protein is present in ethanol-exposed cells, or examining the cellular subregion in which more of the protein is present in ethanol-unexposed cells. The distribution of the protein between different cellular subregions within a cell can also be examined, if desired.

E. Methods and Pharmaceutical Compositions for Reducing Consumption of an Addictive Drug

Another aspect of the invention is a method of reducing consumption of an addictive drug, preferably ethanol. As discussed above, various receptor agonists exert the same effects on the subcellular localization of several proteins as does ethanol, such as, for example, adenosine receptor agonists. Thus, in one embodiment, adenosine A1 receptor agonists, adenosine A2 receptor agonists, non-selective adenosine receptor agonists, and other substances identified by some of the screening methods described above, can be used to help alcoholics or other addicts reduce their consumption of the addictive drug. While not wishing to be held to a particular theory, the inventors believe that because such receptor agonists cause some of the same responses as the addictive drug, the agonists can substitute for the addictive drug. This is similar to the use of methadone for heroin addicts. In a preferred method, consumption is reduced by administering to an animal, preferably a human, an effective amount of a substance which mimics or enhances the effects of the

addictive drug on the subcellular localization of a protein which is distributed differently among the cellular subregions of a DEC versus a DUC. Preferably, the substance mimics or enhances the effects of an addictive drug in some of the screening methods described above. Adenosine receptor agonists are preferred substances.

In another preferred embodiment, adenosine receptor antagonists and other substances identified by some of the screening methods described above can be used to help addicts refrain from consuming an addictive drug. Although other mechanisms may apply, the inventors believe that some of the effects of the addictive drug is due to activation of certain receptors, such as adenosine receptors. Blocking that activation should reduce the effects of the drug. Therefore, in the presence of, *e.g.*, an adenosine receptor antagonist, the addictive drug may not give the consumer the desired response. This should decrease the motivation to consume, thereby reducing consumption. In a preferred method, consumption is reduced by administering to an animal a substance which inhibits the effects of the additive drug on the subcellular localization of a protein having the properties discussed above. Preferably, the animal has chronically consumed the addictive drug but has ceased consumption prior to the administration of the substance. Even more preferably, the animal is not suffering from withdrawal syndrome. Preferably, the substance inhibits the effects of an addictive drug, or inhibits the effects of a receptor agonist exerting the same effects on subcellular localization as an addictive drug, in some of the screening methods described above. Adenosine receptor antagonists are preferred substances.

The present invention also involves methods and medicaments for reducing consumption of an addictive drug through the administration of agonists or antagonists of receptors that, when activated, cause a change in the subcellular distribution of an ethanol-indicative protein. As described in greater detail in Example 7, agonists of dopamine D2 receptors, δ-opioid receptors, cannabinoid receptors, adenosine A1 receptors and adenosine A2 receptors each cause the same translocation of PKA-Cα, δ-PKC and ε-PKC as does ethanol. Thus, the administration of any one of these agonists can be used to reduce consumption of ethanol because each mimics the effects of ethanol on translocation of ethanol-indicative proteins. Where the goal is to reduce ethanol consumption by inhibiting its

cellular effects, the administration of an agonist for one of these receptors is preferred. Reducing consumption of an addictive drug other than ethanol can similarly be achieved by administering an agonist or antagonist of any of these receptors.

Another method of the present invention is based on the inventor's discovery that the following pairs of receptor agonists act synergistically upon the translocation of PKA-Cα: dopamine D2 receptor agonists and δ-opioid receptor agonists; and cannabinoid receptor agonists and dopamine D2 receptor agonists. The following receptor agonists act in synergy with ethanol to translocate PKA-Cα: dopamine D2 receptor agonists; δ-opioid receptor agonists; and cannabinoid receptor agonists. Thus, consumption of an addictive drug can be reduced by administering two active ingredients, one of which inhibits or activates a receptor that is normally activated by the addictive drug of interest and the other of which inhibits or activates (in same direction as other active ingredient) the other member of a synergistic pair. Given the synergy between the two active ingredients, the effective amount of each, when administered together, will be less than when either is administered alone.

Agonists and antagonists of dopamine receptors, opioid receptors, cannabinoid receptors and adenosine receptors are well known to those of skill in the art. For example, without limitation, bromocriptine is a dopamine receptor agonist; sulpiride is a dopamine receptor antagonist; methadone is an opioid receptor agonist; naltrexone is an opioid receptor antagonist; and caffeine is an adenosine receptor antagonist.

Yet another aspect of the invention is a pharmaceutical composition comprising a substance identified by some of the screening methods described above. Preferably, the substance is formulated to comprise, in unit dosage form, an amount effective to reduce consumption of ethanol or other addictive drug during the period of time in which the dosage is active in the body of the animal to which it is delivered.

Pharmaceutically useful substances identified by the methods of this invention may be useful in the form of the free acid, in the form of a salt and as a hydrate. All forms are within the scope of the invention. Basic salts may be formed and are simply a more convenient form for use; in practice, use of the salt form inherently amounts to use of the acid form. The bases which can be used to prepare the salts include preferably those which produce, when combined with the free acid, pharmaceutically acceptable salts, that is, salts whose anions are non-toxic to the

animal organism in pharmaceutical doses of the salts, so that the beneficial properties inherent in the free acid are not vitiated by side effects ascribable to the cations. Although pharmaceutically acceptable salts of the acid compound are preferred, all salts are useful as sources of the free acid form even if the particular salt per se is desired only as an intermediate product as, for example, when the salt is formed only for purposes of purification and identification, or when it is used as an intermediate in preparing a pharmaceutically acceptable salt by ion exchange procedures.

Such substances can be administered to a mammalian host in a variety of forms, *i.e.*, they may be combined with various pharmaceutically acceptable inert carriers in the form of tablets, capsules, lozenges, troches, hard candies, powders, sprays, elixirs, syrups, injectable or eye drop solutions, and the like depending on the chosen route of administration, e.g., orally or parenterally. Parenteral administration in this respect includes administration by the following routes: intravenous, intramuscular, subcutaneous, intraocular, intrasynovial, transepithelial (including transdermal, ophthalmic, sublingual and buccal), topical (including ophthalmic, dermal, ocular, rectal, nasal inhalation via insufflation and aerosol), and rectal systemic. Oral administration is preferred.

Active compounds may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipient and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 6% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 1 and 1000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as polyvinylpyrrolidone, gum tragacanth, acacia, sucrose, corn starch or gelatin; an excipient such as calcium phosphate, sodium citrate and calcium carbonate; a disintegrating agent such as corn starch, potato starch, tapioca

starch, certain complex silicates, alginic acid and the like; a lubricant such as sodium lauryl sulfate, talc and magnesium stearate; a sweetening agent such as sucrose, lactose or saccharin; or a flavoring agent such as peppermint, oil of wintergreen or cherry flavoring. Solid compositions of a similar type are also employed as fillers in soft and hard-filled gelatin capsules; preferred materials in this connection also include lactose or milk sugar as well as high molecular weight polyethylene glycols. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye, flavoring such as cherry or orange flavor, emulsifying agents and/or suspending agents, as well as such diluents as water, ethanol, propylene glycol, glycerin and various like combinations thereof. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

The active compound may also be administered parenterally or intraperitoneally. For purposes of parenteral administration, solutions in sesame or peanut oil or in aqueous propylene glycol can be employed, as well as sterile aqueous solutions of the corresponding water-soluble, alkali metal or alkaline-earth metal salts previously enumerated. Such aqueous solutions should be suitable buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. Solutions of the active compound as a free base or a pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. A dispersion can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal injection purposes. In this connection, the sterile aqueous media employed are all readily obtainable by standard techniques well-known to those skilled in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of

sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique which yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

For purposes of topical administration, dilute sterile, aqueous solutions (usually in about 0.1% to 5% concentration), otherwise similar to the above parenteral solutions, are prepared in containers suitable for drop-wise administration to the eye. The therapeutic compounds of this invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers. As noted above, the relative proportions of active ingredient and carrier are determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice. The dosage of the present therapeutic agents which will be most suitable for prophylaxis or treatment will vary with the form of administration, the particular compound chosen and the physiological characteristics of the particular

patient under treatment. Generally, small dosages will be used initially and, if necessary, will be increased by small increments until the optimum effect under the circumstances is reached. Oral administration requires higher dosages. The compounds are administered either orally or parenterally, or topically as eye drops. Dosages can be readily determined by physicians using methods known in the art, using dosages typically determined from animal studies as starting points.

IV. Methods Relating to the Alteration of Cellular Levels of a Protein in a Cell Exposed to an Addictive Drug

In another aspect, the present invention provides a method of measuring the exposure to ethanol or other addictive drugs of a sample containing at least one cell, the method comprising measuring, either quantitatively or qualitatively, the amount of a protein or polypeptide, which amount is dependent on exposure of the cell to ethanol or other addictive drugs, and determining the amount of the protein in the cell. In one preferred embodiment, the polypeptide may be the type I regulatory subunit (RI) of PKA where a reduction, for example a 20% to 50% reduction compared to unexposed control cells, is indicative of exposure to ethanol or other addictive drugs. Alternatively, the detectable amount of the protein heat stable protein kinase inhibitor (PKI) may be measured and correlated with exposure to an addictive drug. In another preferred embodiment, the increase of the detectable amount of the α-, δ-, or the ∈-subunit of PKC may be determined and correlated with exposure. The determination may be carried out by any of a variety of measurement methods well known to persons of ordinary skill in the art of molecular biology, such methods include ELISA, radioimmunoassay, western blot analysis, and the like.

Substances can be screened for their ability to alter or mimic the effects of ethanol or other addictive drugs on the cellular levels of a protein, such as an ethanol indicative protein by performing assays in which the quantity of *e.g.*, PKA RI, α-PKC, δ-PKC, ∈-PKC, or PKI is measured.

V. Kits

Another aspect of the invention is to provide kits for carrying out the subject methods. Kits generally contain one or more reagents necessary or useful for practicing the methods of the invention. Reagents may be supplied in pre-measured

units so as to provide for uniformity and precision in test results. The invention provides kits for determining the exposure of cells to ethanol by means of measuring the amount of RI, αPKC, δ-PKC, or ∈-PKC. These RI, αPKC, δ-PKC, or ∈-PKC measurement kits comprise a stain specific for RI, αPKC; δ-PKC, or ∈-PKC. The RI, αPKC, δ-PKC, or ∈-PKC measurement kits may further comprise one or more of the following items' additional reagents for the detection of RI, αPKC, δ-PKC, or ∈-PKC complexed with the stain; positive controls for RI, αPKC, δ-PKC, or ∈-PKC; negative controls for RI, αPKC, δ-PKC, or ∈-PKC; RI, αPKC, δ-PKC, or ∈-PKC solutions of known concentration; equipment for obtaining tissue samples, and the like.

Kits for determining the exposure of cells to ethanol or other addictive drugs by means of determining the intracellular localization of, *e.g.*, an ethanol indicative protein, such as, for example, PKA Cα, PKA RIα, PKA RIIβ, α-PKC, δ-PKC or ∈-PKC preferably comprise a stain specific for the protein or for a gene product the transcriptional regulation of which is mediated by the protein. Such kits may further comprise one or more of the following items: additional reagents required for the detection of a protein that has complexed with the stain, a reference standard(s), stain specific for a cellular subregion, equipment for obtaining tissue samples, instructions for use, and the like. Suitable reference standards include positive controls, negative controls, photographs of such controls, tabulated or graphed data of such controls, and the like. The kits may further comprise instructions for carrying out the methods described above, preferably printed instructions. For example, the instructions can describe detecting the amount of an ethanol indicative protein in a first cellular subregion of a test cell. Suitable stains include, but are not limited to, stains specific for PKA Cα, PKA RIα, PKA RIIβ, δ-PKC, and ∈-PKC.

The invention also provides kits for testing compounds for their ability to modulate cellular responses to ethanol using the assay methods of the invention. Such kits are essentially the same as the kits described above for the measurement of cellular localization or protein levels. However, such kits may further comprise a polynucleotide comprising a reporter gene and/or a host cell line useful for the detection of changes in localization. Suitable cell lines are described above. Preferably the host cell line has been transfected with the reporter gene as discussed above. Preferably, a kit using a stain to (1) detect cellular localization of a protein in

a cell exposed to an addictive drug, or (2) identify a substance that alters or mimics the effects of an addictive drug on cellular localization, further comprises printed instructions for comparing the localization of the stain in the test cell to the localization of the stain in a control cell of the same cell type. The control cell can be an EEC or an EUC or both. The printed instructions can include a description of localization of one or more ethanol indicative proteins in one or more different cell types. For example, the instructions can include information in the form of a table, such as Table 1. The instructions can also include photographs of such cells, if desired.

Kits using a stain specific for a gene product, the production of which is regulated by a protein which is localized in a different cellular subregion in an EEC than an EUC (*e.g.*, PKA-Cα), or a polynucleotide encoding the gene product, preferably include printed instructions for comparing the production levels of the gene product in a test cell to the production levels in a control cell. Such kits may also include other reagents necessary for detecting the stain, such as, for example, reagents required for the detection of luciferase activity, such as luciferin, ATP and Co-enzyme A, or, where the stain is a polynucleotide probe, a radioisotope or biotin.

EXAMPLES

Example 1: Ethanol Induced Translocation of the Cα Catalytic Subunit of PKA

NG108-15 cells were plated onto single chamber slides in a defined medium at a density of approximately 40,000 cells/slide. The techniques and media used for growing the cells are not critical, and are known to those skilled in the art. Suitable techniques are described in Gordon *et al.*, 1986, *Proc. Natl. Acad. Sci USA* 83:2105. The cells were maintained for an additional forty eight (48) hours in the defined media or the defined media containing various concentrations of ethanol (*e.g.*, 25, 50, 100, 200 mM ethanol). The media were replaced by fresh media (with or without ethanol) daily and the slides were wrapped in parafilm to prevent ethanol evaporation. The cells were fixed with methanol on a cooled surface for two (2) to three (3) minutes, and the slides were then immersed twice for five (5) minutes each in phosphate buffered saline (PBS) on ice. After that, the cells were incubated with blocking buffer (1% normal goat serum in PBS containing 0.1% Triton-X-100) at 4°C for six (6) to twelve (12) hours and then incubated with primary antibody solution for

forty eight (48) hours at 4°C in a humidified chamber. The primary antibody solution was prepared from primary antibodies raised in response to Cα (available from Transduction Laboratories and other companies) diluted in PBS containing 0.1% Triton X-100 and 2 mg/ml fatty acid free bovine serum albumin. The slides were washed as before and incubated in the appropriate FITC (fluoresceinisothiocyanate)-conjugated secondary antibody diluted in the same solution at 1:1000.

After forty-eight (48) hours, the slides were washed and coverslipped using Vectashield™ mounting medium (Vector Labs). The images in panels A and B of FIG. 1 were made using a BIORAD™ 1024 confocal microscope. The images in panels C and D of FIG. 1 were made using a Leica™ DMBR microscope equipped with a fluorescein filter.

To determine reversibility of exposure to ethanol (results shown in panel C of FIG. 1), the cells were exposed to media containing 200 mM ethanol for forty eight (48) hours then washed three times with fresh media containing no ethanol and incubated without ethanol for an additional forty eight (48) hours. To determine specificity of binding of the stain (results shown in panel D of FIG. 1), 0.1 mg/ml purified catalytic subunit of PKA was added to the primary antibody solution two (2,) hours prior to incubation with the fixed cells.

To obtain the numerical data shown in FIG. 2, random fields were selected on the slide and the cells within the field classified as either having primarily Golgi staining or primarily nuclear staining for Cα. Although using only two types of classification simplifies scoring of cells, it may be desirable to classify cells into a plurality of classification groups dependent on the degree of staining in each of several locations. In some instances, it may even be desirable to provide continuous variable classification of each cell, for example, if image intensity is measured at a given point in a cell. At least five (5) fields were classified for a total of at least one hundred (100) cells per slide. The observer was blind to the experimental condition of the slides. Data points are the mean +/- SEM (standard error of the mean copy) of four (4) experiments, $*p < 0.05$.

The results obtained can be summarized as follows: NG108-15 cells, forming a control which had not been exposed to ethanol, were stained with the specific stain. Cα was found in the perinuclear Golgi area in approximately 80% of the control cells, as depicted in panel A of FIG. 1. In the remaining 20% of the control cells, Cα was

found predominately in the nucleus and cytoplasm. This localization of the Cα subunit to the Golgi has been previously observed by Nigg and co-workers. Nigg *et al.*, 1985, *EMBO J.* 4:2801-2806. Cellular localization of Cα was further confirmed by co-localization with Golgi-specific markers, including mannosidase II, and ceramide. Nigg *et al., supra.*

Other cell samples were exposed to ethanol of varying concentrations (25, 50, 100, 200 mM), for a period of forty eight (48) hours, and the assay repeated. The results are shown in FIG. 2. As can be seen from FIG. 2, 75 % of cells treated with 200 mM ethanol showed predominant localization of Cα in the nucleus. A micrograph of these cells is shown in panel B of FIG. 3. The results of these investigations serve as a reference to which a sample of cells suspected of being exposed to ethanol can be compared. To provide a screen for a drug or therapeutic agent to discover whether it has any effect on the cellular effects of ethanol, the above procedure can be repeated with the drug present in the growth medium in addition to alcohol. The cells can be pre-incubated with the drug, if desired. Since 100 mM ethanol is found to have a noticeable effect, and 200 mM ethanol is found to have a marked effect on the localization of Cα PKA, a screen for a drug can be provided employing a single growth medium preferably having a ethanol concentration of the order of at least 100 mM, and more preferably of 200 mM, or more. Of course, several growth media containing differing amounts of ethanol may be used, as described above, to investigate efficacy of the drug at varying levels of ethanol concentrations. This may be particularly useful for investigating the effects of a drug on long-term low-level exposure to ethanol.

The results from a control sample can be stored so that when screening for a particular drug, it is not necessary to conduct a control experiment each time. However, it is often desirable to conduct a control experiment whenever a drug is screened, to compensate for variations in other factors which may affect the results.

The above procedure is sufficient to form the basis for a screen. Additional factors having an effect on the localization of PKA Cα and activity of other PKA subunits have been identified. The following information may therefore be of assistance in assessing how the screening method may be affected by external factors.

Reversibility of the localization is demonstrated by panel C of FIG. 1, which is a micrograph of a similar sample forty eight (48) hours after withdrawal from the

ethanol. As can be seen, the majority of the Cα had returned to the Golgi apparatus. In a screen for a drug, therefore, it is important to classify the cells relatively soon after removal from the medium, usually within forty eight (48), preferably within twelve (12) hours, preferably immediately. Similarly, samples taken from patients should be classified soon after removal.

To test for specificity of the stain, the stain was preabsorbed by purified Cα prior to staining, and as can be seen from panel D of FIG. 1, virtually no staining resulted, indicating that the polyclonal antibody stain is specific to Cα.

The time dependence of the localization of the Cα and the effects of other substances were investigated (FIG. 4) as follows: NG108-15 cells were cultured as described above. After two (2) days in defined media, cells received media containing either 200 mM ethanol or 1 μM forskolin for various lengths of time, as described below. Control cells received fresh media alone at the same time points. All slides were fixed four (4) days after plating and stained for Cα as described above. Similar experiments were performed using 25 mM and 50 mM ethanol over a period of four (4) to five (5) days; comparable results were obtained.

To obtain the numerical results, depicted in FIG. 4, fields were selected as described above, and cells were scored as either having Cα staining confined primarily to the Golgi or extensive staining outside of the Golgi. Data points are the mean +/- SEM of three (3) experiments.

The results will now be discussed with reference to FIG. 3 and FIG. 4. Panel A of FIG. 3 shows that the Cα was localized at the Golgi in the control cells, as before, and panel B of FIG. 3 shows that after forty eight (48) hours exposure to ethanol, Cα staining was found in the nucleus. This confirms the earlier described results.

Stimulation by 10 μM PGE, resulted in diffuse staining throughout the cell, as shown in panel C of FIG. 3. Similar results were achieved by treatment with 1 μM forskolin, as shown in panel D of FIG. 3. Maximal translocation of Cα away from the Golgi occurred after about thirty (30) minutes, with forskolin or PGE, which is when the micrographs shown in panels C and D of FIG. 3 were taken. This was followed by a return of staining to the Golgi for forskolin but not for ethanol (FIG. 4).

This is clearly contrasted with the effects of exposure to ethanol, where (see FIG. 4) after a relatively brief exposure to ethanol (from 30-60 minutes), little change

in the localization of Cα was detected. After six (6) hours exposure to ethanol, translocation from the Golgi to the nucleus of the Cα was apparent, and after twelve (12) hours, most of the cells had developed prominent nuclear staining, with a corresponding decrease in Golgi staining. This staining remained through forty eight (48) hours of exposure to ethanol.

Thus, in screening for the effect of drugs on ethanol-induced altered long-term localization of Cα, best results will be obtained if the cells are left in the growth medium for at least about twelve (12) hours, and more preferably about forty eight (48) hours (two (2) days). The effects of ethanol exposure can be distinguished from the effects of other substances which produce a relatively temporary reversible change in Cα localization. Ethanol also causes an increased amount of Cα to be detected in the nucleus about 10 min. after exposure, which disappears by about 30 min.

The investigation of movement and activity of other PKA subunits particularly type I (RI) and type II (RII) regulatory subunits was investigated. Stains using monoclonal antibodies, prepared by conventional immunocytochemistry techniques, analogous to those described above for the Cα subunit were used. The RII subunit and the RI subunit were detected primarily on the Golgi apparatus. Ethanol was not found to have any significant effect on the localization of the RI subunit within the NG108-15 cell, but the RII subunit translocated to the nucleus.

It was, however, found that the amount of RI subunit was decreased by exposure to ethanol. Results of a western blot analysis showing that exposure to 200 mM ethanol for forty eight (48) hours had no effect on the amount of Cα subunit, but produced a decrease of about 40% (43 +/- 3 %) in the RI subunit are shown in FIG. 5. Thus, an alternative assay procedure can be provided by measuring the amount of PKA RI.

With the above results, the effects of ethanol on NG108-15 cells can thus be clearly identified. To produce a screen for therapeutic agents or drugs that modulate the cellular effects of exposure to ethanol, these cells can be exposed to ethanol in the presence of a drug whose activity is to be investigated, and the results compared to those obtained from cells exposed to ethanol in the absence of the drug.

Example 2: Ethanol Induced Translocation of δ-PKC and ∈-PKC

Immunohistochemistry of δ-PKC in NG108-15 cells, grown in defined medium, shows predominant Golgi staining (FIG. 6); approximately 70% of these cells show Golgi staining (Table 2). After 48 hours ethanol exposure (200 mM EtOH), δ-PKC is localized to the perinucleus and nucleus and absent from the Golgi (FIG. 6 and FIG. 7). More than 90% of the cells show perinuclear and nuclear staining (Table 2). The specificity of the fluorescence staining for δ-PKC is indicated by the absence of staining when the anti-δ antibody is preabsorbed with the immunizing peptide before labeling of the cells (FIG. 7). These results suggest that ethanol exposure causes translocation of δ-PKC from the Golgi to the perinucleus and nucleus since there is little δ-PKC remaining in the Golgi area (Table 2) after ethanol exposure.

Ethanol also alters localization of ∈-PKC. In naive cells, ∈-PKC is localized to the perinucleus in more than 90% of the cells (FIG. 8, FIG. 9 and Table 2), with no measurable cytoplasmic staining. After ethanol exposure, ∈-PKC staining is observed throughout the cytoplasm in greater that 90% of the cells (FIG. 8, FIG. 9 and Table 2); perinuclear staining is still present in more that 90% of the cells (FIG. 8, FIG. 9 and Table 2). The staining for ∈-PKC appears to be specific since no staining is observed when the anti-∈ antibody is preabsorbed with immunizing peptide (FIG. 9). Ethanol-induced altered localization of δ-PKC and ∈-PKC is also observed after exposure to 25 mM ethanol for four (4) days (FIG. 7 and FIG. 9).

Ethanol-induced altered localization of PKC isozymes could be similar to that induced by phorbol esters or hormones or to sites different from these latter activators. Naive NG108-15 cells were, therefore, incubated in for ten (10) min. in 100 nM PMA, to then determine localization of δ-PKC and ∈-PKC. On activation by PMA, the δ isozyme is mainly translocated to the perinucleus (FIG. 6), suggesting that ethanol-induced translocation of δ-PKC is to sites similar to those occupied after activation by PMA. In contrast, translocation of ∈-PKC due to PMA activation results in nuclear and perinuclear localization of this isozyme, different from ethanol-induced translocation to the cytoplasm (FIG. 8).

The effects of ethanol on NG108-15 cells can thus be clearly identified by determination of the localization of δ-PKC and ∈-PKC. To produce a screen for

therapeutic agents or drugs that modulate the cellular effects of exposure to ethanol, these cells can be exposed to ethanol in the presence of a drug whose activity is to be investigated, and the results compared to those obtained from cells exposed to ethanol in the absence of the drug.

Table 2

δ-PKC (% cells)				∈-PKC (% cells)			
Golgi staining		Perinuclear/nuclear staining		Perinuclear staining		Cytoplasmic staining	
Control	EtOH	Control	EtOH	Control	EtOH	Control	EtOH
74±7	2±2	2±2	93±5	95±2	94±3	5±2	94±2
(n=5)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)	(n=5)

- The percentage of particular immunostaining was obtained from five (5) experiments, each experiment was counted in four (4) fields per slide, three (3) or four (4) different slides in the same experience.

Example 3: Altered Localization of PKA Subunits in Lymphocytes and Neutrophils Exposed to Ethanol *in vitro*

Non-alcoholic controls and chronic alcoholics completed an ICD10 questionnaire (a standard means for diagnosis of alcoholism) and were interviewed about their lifetime alcohol consumption. A blank questionnaire is provided in Table 3. Subjects who used other addictive drugs were excluded from the study. Blood alcohol levels were measured at the time of sampling using Alco-screen dipsticks. Routine CBC, triglyceride levels and liver function tests were carried out by the Clinical Hematology Laboratory at San Francisco General Hospital.

Table 3

Alcohol Dependence Questionnaire

Have you experienced any of the following more than twice in the past year:

Compulsion			
1)	Had a strong desire or urge to drink	Yes	No
2)	Felt powerless over your drinking	Yes	No
3)	Needed a drink so badly you couldn't think of anything else	Yes	No
Impaired Control			
4)	Tried to cut down or stop drinking and found you couldn't do it	Yes	No
5)	Wanted to cut down or stop your drinking and found you couldn't do it	Yes	No
6)	Ended up drinking much more than you intended to	Yes	No
7)	Found it difficult to stop drinking once you had started	Yes	No
8)	Kept on drinking for a longer period than you had intended to	Yes	No
9)	Started drinking even though you hadn't intended to	Yes	No
Withdrawal			
10)	Been sick or vomited after drinking or the morning after	Yes	No
11)	Felt depressed, irritable or nervous after drinking or the morning after	Yes	No
12)	Found yourself sweating heavily or shaking after drinking or the morning after	Yes	No
13)	Heard or seen things that weren't really there after drinking or the morning after	Yes	No
14)	Taken a drink to keep yourself from shaking or feeling sick either after drinking or the morning after	Yes	No
Tolerance			
15)	Found that the same amount of alcohol had less of an effect than before	Yes	No
16)	Found that you had to drink more than you once did to get the same effect	Yes	No
Given up activities for drinking			
17)	Given up or cut down on activities or interests like sports or associations with friends, in order to drink	Yes	No

18)	Lost ties with or drifted apart from a family member or friend because of your drinking	Yes	No
19)	Had your chances for promotion, raises or better jobs hurt by your drinking	Yes	No

Continued use despite harmful consequences

20)	Continued to drink alcohol even though it was a threat to your health	Yes	No
21)	Kept drinking even though it caused you emotional problems	Yes	No

Clinician: In order for a diagnosis of alcohol dependence (based on the ICD – 10) to be made, at least one item in each of three separate categories must be endorsed.

Isolation of Lymphocytes and Neutrophils

Blood (50 ml) from non-alcoholic controls and alcoholics were drawn into heparinized test tubes and cell types separated on a Ficoll Histopaque step density gradient (1.077/1.119 g/ml) according to English et al., *J. Immunol. Methods* 5:249-252 (1974). The tubes were centrifuged at 700 x g for 30 min at room temperature. Two bands were collected; the upper band on top of the Ficoll contained platelets, monocytes and lymphocytes (lymphocyte fraction) and the lower band at the interface of the two Ficoll layers contained granulocytes and erythrocytes (neutrophil fraction). Contaminating erythrocytes were eliminated from the cells in the lower band by hypotonic shock. This treatment did not affect localization of PKA subunits. Cells in each band were diluted 1:10 in phosphate-buffered saline (PBS) and washed three times by centrifugation at 200 x g. This step removed contaminating platelets from the lymphocyte fraction. 95% of the cells in each subpopulation were neutrophils or lymphocytes, respectively. Cell viability, determined by 0.4% Trypan Blue staining, was greater than 98% for both populations just after isolation and approximately 85% six hours later.

Cell Treatment

Suspensions of lymphocytes (8 X 10^5 cells/ml) or neutrophils (5 X 10^5 cells/ml) from non-alcoholics were incubated in the absence or presence of various concentrations of ethanol for the indicated times. 150 µl of cell suspension was added to individual cytofunnels and the incubation stopped by centrifuging the cells in a Shandon II cytospin centrifuge for 3 min at 1000 rpm onto Falcon double chamber glass tissue culture slides in two stages. Neutrophils were spread at the bottom and lymphocytes on the top of the slides. The slides were then separated from the funnels

and air dried before the cells were fixed with methanol at room temperature for 15 min.

Immunocytochemistry

Blocking solution (1% normal goat serum, 0.1% Triton x-100 in PBS) was added to fixed cells and the slides incubated for two hours at room temperature. Buffer was then aspirated and cells were incubated overnight at 4°C with primary antibody to either RIα, RIIα, RIβ, Cα, Cβ, or Cγ (diluted 1:100 in PBS containing 0.2% fatty acid free bovine serum albumin (FAF-BSA)), and 0.1% Triton x-100. Slides were then washed 3 times with PBS, placed in humid chambers, and goat-anti-rabbit antibody conjugated to fluorescein isothiocyanate (diluted 1:250 in PBS containing FAF-BSA) for two hours at room temperature. After washing the slides 3 times in PBS, buffer was aspirated and one drop of Vectashield fluorescent mounting solution was added to each slide before covering and incubating overnight at 4°C.

Intracellular localization of PKA subunits was evaluated in 0.5 mm sections using a Bio-Rad 1024 confocal microscope (60 X objective, total magnification 600X) and LaserSharp software. Images were further analyzed using NIH Image.

Localization in Neutrophils

All three catalytic subunits of PKA (Cα, Cβ, Cγ) were present in the cytoplasm of neutrophils; addition of either 200 mM ethanol or 100 nM forskolin had no effect on localization of the catalytic subunits. RIα was also localized to the cytoplasm. However, after acute treatment with ethanol or forskolin, RIα translated from the cytoplasm to the nucleus. See, for example, FIG. 10A and FIG. 10C. After acute ethanol exposure, some RIα diffused out of the cells, most likely due to cellular membrane disruption as a result of the high sensitivity of neutrophils to *in vitro* treatments. This did not occur in control cells not receiving ethanol or forskolin. In contrast to RIα, ethanol and forskolin had no effect on the localization of RIIα. Ethanol or forskolin did not alter RIβ localization in neutrophils. No staining was observed for any antibodies after preincubation for two hours in the presence of a ten-fold excess of the respective immunogenic peptide prior to incubation with the leukocytes, indicating the specificity of staining.

Translocation of RIα to the nucleus in neutrophils was observed after 10 min of exposure to 200 mM ethanol. At lower, more physiological concentrations of

ethanol, translocation of RIα occurred after 2 hours with 50 mM ethanol or after 4 hours with 25 mM ethanol.

Localization in Lymphocytes

Ethanol exposure caused a dramatic translocation of Cα from the cytoplasm to the nucleus in lymphocytes. Translocation of Cα to the nucleus was also observed at low concentrations of ethanol (25 and 50 mM ethanol), beginning after two hours with 50 mM ethanol or after three hours of incubation with 25 mM ethanol. At lower concentrations of ethanol, a specific pattern of Cα staining in the nucleus can be seen, suggesting that localization in the nucleus is not due merely to diffusion but to binding to specific sites in the nucleus. Localization of Cβ, Cγ, and RIα in lymphocytes was not altered by ethanol. In human lymphocytes, we did, however, observe a partial translocation of the RIβ subunit from cytoplasm to nucleus. The RIIα subunit is localized in the nucleus of lymphocytes from non-alcoholic controls even without any treatment. Thus, *in vitro* ethanol exposure alters the localization of RIα and Cα in neutrophils and lymphocytes respectively.

Example 4: Altered Localization of PKA Subunits in Lymphocytes and Neutrophils from Actively Drinking Alcoholics

Lymphocytes and neutrophils were isolated from actively drinking alcoholics and were examined by immunocytochemistry, as described above.

RIα was localized in the nuclei of untreated neutrophils from alcoholics as shown in FIG. 10B. This is in agreement with the localization of PKA RIα in cells from non-alcoholic controls exposed to ethanol *in vitro* and suggests that, in neutrophils of alcoholics, PKA RIα is translated to the nucleus *in vivo* due to ethanol exposure. Further *in vitro* ethanol treatment of neutrophils from alcoholics did not result in any change in localization of RIα, as shown in FIG. 10D. RIIα and RIβ were localized to the cytoplasm in leukocytes from alcoholics either in the absence or presence of *in vitro* ethanol treatment, similar to results from non-alcoholic controls.

In vivo ethanol exposure also caused translocation of Cα. As shown in FIG. 11B, Cα was localized to the nucleus in untreated lymphocytes of alcoholics, similar to its localization in lymphocytes from non-alcoholic controls following acute *in vitro* ethanol exposure. Cα in lymphocytes from alcoholics remained in the nucleus after *in vitro* treatment with ethanol. Hence, there is a marked difference in the localization

of RIα in neutrophils and Cα in lymphocytes between alcoholics and non-alcoholic controls.

Correlation between Alcoholic State and PKA Subunit Localization

In order to determine whether there is a correlation between alcoholic state and PKA subunit localization, state, daily alcohol consumption, lifetime alcohol consumption, blood alcohol levels, total triglycerides and liver function profile of all participants in the study were obtained (Table 4). A correlation between alcoholic state and nuclear localization of RIα and Cα in neutrophils and lymphocytes, respectively, was found in seven out of eight alcoholics; in one alcoholic, nuclear localization of Cα and not RIα was correlated. One putative alcoholic showed no correlation, but he did not meet either of the ethanol consumption criteria. All non-alcoholic controls showed only cytoplasmic localization of RIα and Cα in both cell types. The correlation between nuclear localization of RIα and Cα and alcoholic state was statistically significant as evaluated by frequency distribution tests using Fisher's exact P value (P<.0032 for neutrophils and P<.0014 for lymphocytes.

Table 4

Correlation between Alcoholic Consumption and Enzyme Translocation

Patient ID	Alco screen	Icoholic*	Alcohol consumption last 12 h	Daily alcohol consumption	Liver functions †	Triglyceride level	Lifetime alcohol consumption	RIα in nucleus	Cα in nucleus ( )#
247	-	?	None	140 ml	all normal	160 mg/dl	597 Kg	-	-
351	+	+	240 ml	307 ml	all abnormal	560 mg/dl	1267 Kg	+	+ (2)
255	+	+	235 ml	350 ml	all abnormal	172 mg/dl	969 Kg	+	+ (2)
253	+	+	151 ml	276 ml	all normal	274 mg/dl	2106 Kg	+	+
259	-	+	52 ml	310 ml	all normal	71 mg/dl	1042 Kg	+	+ (3)
267	-	+	None	262 ml	all normal	65 mg/dl	752 Kg	+	+
269	-	+	60 ml	280 ml	all normal	339 mg/dl	2405 Kg	+	+ (2)
271	-	+	149 ml	190 ml	all normal	73 mg/dl	2717 Kg	+	+
265	-	+	None	80 ml	all normal	270 mg/dl	2189 Kg	-	+
312	-	-	None	0 ml	all normal	140 mg/dl	81 Kg	-	- (2)

Patient ID	Alco screen	Icoholic*	Alcohol consumption last 12 h	Daily alcohol consumption	Liver functions †	Triglyceride level	Lifetime alcohol consumption	RIα in nucleus	Cα in nucleus ( )#
226	-	-	None	26 ml	all normal	185 mg/dl	231 Kg	-	-
244	-	-	None	18 ml	all normal	210 mg/dl	146 kg	-	- (2)
306	-	-	None	0 ml	all normal	180 mg/dl	34 Kg	-	- (4)
144	-	-	None	0 ml	all normal	120 mg/dl	28 Kg	-	-
206	-	-	None	0 ml	all normal	200 mg/dl	14 Kg	-	-
310	-	-	None	0 ml	all normal	131 mg/dl	10 Kg	-	- (3)

( ) = # of times blood drawn from subject

Example 5: Screening for Substances that Inhibit Ethanol-Induced Translocation of ∈-PKC

NG 108-15 or CHO cells were pre-incubated in the presence or absence of the indicated inhibitor for 30 min. (except for G_F which was pre-incubated for 1 hour), then further incubated in the absence or presence of 200 mM ethanol for 30 min.

Table 5

Inhibitors of Ethanol-Induced Translocation of ∈-PKC

Compound	Class	Predominant ∈-PKC Localization
None		perinucleus
Ethanol 200 mM	n/a	cytoplasm
Ethanol + 15 ng/ml Pertussis toxin	Inhibitor of G_i, G_o and G_z mediated functions	perinucleus
Ethanol + $2x10^{-4}$ M MDL 12, 330A Hydrochloride	Adenylyl cyclase inhibitor	perinucleus
Ethanol + 20 μM R_p-cAMPS	PKA antagonist	perinucleus
Ethanol + 10 μM Et-18-OCH_3	PLC inhibitor	perinucleus
Ethanol + 1 μM U 73122	PLC inhibitor	perinucleus
Ethanol + 100 nM MG_F (Bisindoylmaleimide I)	PKC inhibitor	perinucleus
Ethanol + 40 nM Gö	PKC inhibitor	perinucleus

Example 6: Screening for Substances that Alter Luciferase Expression in Transfected Cells Exposed to Ethanol

Luciferase is used as a reporter gene in a transient expression system to identify substances that alter the effects of ethanol on CRE-regulated gene expression. Cells are cultured as described in Dohrman et al., *Proc Natl Acad Sci USA* (1996) 93:10217-10221, and are transfected using Effectene (Qiagene) as described by the manufacturers. Twenty-four hours after transfection the cells are treated with 200 mM ethanol for 30 min. to 36 hr. Luciferase activity is measured using Luciferase Assay System (Promega) and normalized to β-galactosidase activity.

Such cells can be used as described above to screen substances for their ability to alter or mimic the cellular affects of ethanol.

Ethanol Dependent Luciferase Expression in NG108-15 Cells Transfected with GAL4-Luciferase Fusion Construct

NG108-15 cells are co-transfected with a GAL4-CREB fusion construct and with a second construct containing the DNA binding site of GAL4 fused to luciferase as a reporter gene. As shown in FIG. 12, ethanol exposure increased the expression of luciferase approximately 2-fold. The ethanol-induced increase in luciferase activity induced was inhibited by Rp-cAMPS but not by the CaMK and PKC inhibitors KN-62 and GF, respectively (FIGS. 15 and 16B). These data demonstrate that phosphorylation and activation of CREB are required for ethanol-induced increases in CRE-mediated luciferase transcription.

Ethanol Dependent Luciferase Expression in NG108-15 Cells Transfected with CRE-Luciferase Fusion Construct

NG108-15 cells were co-transfected with a fusion construct containing the cAMP response element (CRE) fused to luciferase as a reporter gene (Stratagene). As shown in FIG. 12, ethanol exposure increased the expression of luciferase more than 2-fold. As shown in FIG. 13, an increase (29±6% above control) in luciferase activity was first apparent after a 4 hour exposure to 200 mM ethanol and a second increase (79±4% above control) occurred after 14 hours of exposure to 200mM ethanol. A 14-hour exposure to 50mM ethanol also resulted in an increase (about 25%) in luciferase activity.

NG108-15 cells having the CRE-luciferase reporter gene were co-incubated with 200mM ethanol and either an adenosine receptor antagonist (BW A1434U, 10μM, Glaxo Wellcome) or a selective PKA inhibitor (Rp-cAMPS, 20μM, Biolog Life Science Institute, La Jolla, CA). As shown in FIG. 14, the adenosine receptor antagonist blocked the peak in luciferase activity normally observed after 4 hours of ethanol exposure and the PKA inhibitor completely inhibited both the early and late phases of ethanol-induced increases in luciferase expression. The essential role of PKA in mediating ethanol-induced changes in luciferase expression was further demonstrated by the finding that H-89 (10μM, Calbiochem, San Diego, CA), another selective PKA inhibitor, and DN-RIα (Clegg, C. H., Correll, L. A., Cadd, G. G., and McKnight, G. S. (1987) *J Biol Chem* **262**(27), 13111-9)), a dominant negative form of PKA-RIα that has mutations at amino acid 200 in site A and amino acids 324 and 332 in site B and interferes with PKA activation, each inhibit the increase in luciferase expression that otherwise occurs after14 hours of exposure to 200mM ethanol (FIG.

15). CREB phosphorylation and activation are also essential for ethanol-induced increases in luciferase expression – a mutant form of CREB (CREB-M1, Dr. M.E. Greenberg) which, on account of the replacement of Ser-133 by alanine, can bind CRE but cannot be phosphorylated or activated, completely prevented ethanol-induced increases in luciferase activity at 14 hours (FIG 16A). Since the ERK/MAPK pathway may regulate CREB phosphorylation and CRE-mediated gene expression, NG108-15 cells having the luciferase construct were co-incubated with 200mM ethanol and a MEK inhibitor (PD98059 (2μM, Calbiochem, San Diego, CA) or U0126 (1μM, Calbiochem, San Diego, CA)) or a dominant negative MEK construct (DN-MEK, Dr. R. Seger) in which Lys-97 was replaced by alanine. As shown in FIG. 15, the MEK inhibitors and the dominant negative MEK construct all eliminated the increase in luciferase activity normally seen after 14 hours of ethanol exposure. Thus, long term changes in ethanol-induced, CRE-regulated gene expression are dependent upon ERK/MAPK signaling.

Ethanol Dependent Luciferase Expression in CHO Cells Transfected with CRE-Luciferase Fusion Construct

CHO cells are transiently transfected with Gal4-CREB and Gal4-Luciferase or with CRE-Luciferase (Stratagene) utilizing Effectene (Qiagene) as described the manufacturers. Twenty-four hours after transfection the cells are treated with 200mM ethanol for 30 min. to 36 hr. Luciferase activity is then measured using Luciferase Assay System (Promega) and normalized to β-galactosidase activity.

Drug Dependent Luciferase Expression in NG108-15 Cells Transfected with CRE-Luciferase Fusion Construct

NG108-15 cells were co-transfected with a fusion construct containing the cAMP response element (CRE) fused to luciferase as a reporter gene (Stratagene). Incubation of these cells for four hours with an opioid agonist (10nM enkephalin (D-ala2,D-leu5) ("DADLE")) caused a forty percent (40%) increase in luciferase expression that closely resembled the ethanol-induced changes after four hours.

Example 7: Effects of Various Receptor Agonists and Antagonists on ∈-PKC, δ-PKC or PKA Cα Translocation

To determine whether addictive drugs other than ethanol would cause translocation of some proteins, we examine the effects of various receptor agonists and antagonists on ε-PKC, δ-PKC or PKA Cα translocation in NG108-15 and CHO cells expressing the applicable receptor. As shown below in Table 6, δ-opioid receptor agonists, cannabinoid receptor agonists, and dopamine receptor agonists do cause translocation in such NG108-15 cells, but muscarinic agonists and $\alpha 2_B$ adrenergic receptor agonists do not, suggesting that addictive drugs other than ethanol also cause translocation. Pre-treatment with 10μM spiperone, a dopamine receptor antagonist, blocked NPA-induced translocation of δ-PKC, ε-PKC and PKA Cα and had no effect upon ethanol-induced translocation of δ-PKC, ε-PKC and PKA Cα. Similar translocation of δ-PKC, ε-PKC and PKA Cα was observed in dopamine D2 receptor-expressing CHO cells after treatment with ethanol, dopamine receptor agonist NPA and/or dopamine receptor antagonist spiperone. Adenosine is also implicated in some behavioral effects of ethanol. Interestingly, adenosine receptor agonists cause translocation of ∈-PKC and adenosine receptor antagonists prevent ethanol-induced translocation of PKA-Cα and ε-PKC but have no effect on ethanol-induced translocation of δ-PKC.

Table 6

Translocation of ∈-PKC, δ-PKC or PKA Cα by G_i- or G_s- Coupled Receptors

Treatment	Protein	Predominant Localization
None	ε-PKC	Perinucleus
200mM Ethanol	ε-PKC	Cytoplasm
50 nM D2 Dopamine receptor agonist NPA	ε-PKC	Cytoplasm
1 μM δ-Opiate receptor agonist D-ala-D-leu-enkephalin	ε-PKC	Cytoplasm
2 μM Cannabinoid receptor agonist R(+) methanindamide	ε-PKC	Cytoplasm
20 μM Muscarinic acetylcholine receptor agonist (Carbachol)	ε-PKC	Perinucleus
10^{-5} M $\alpha 2_B$ Adrenergic receptor agonist (UK14304)	ε-PKC	Perinucleus
20 nM Adenosine Al receptor agonist CCPA (2-chloro-N^6-cyclopentyladenosine)	ε-PKC	Cytoplasm
100 nM Adenosine A2 receptor agonist CGS-21680 HC1	ε-PKC	Cytoplasm
10μM Dopamine receptor antagonist spiperone + 50 nM D2 Dopamine receptor agonist NPA	ε-PKC	Perinucleus
10μM Dopamine receptor antagonist spiperone + 200 mM ethanol	ε-PKC	Cytoplasm
10 μM Adenosine receptor antagonist BW 1434 + 200 mM ethanol	ε-PKC	Perinucleus
None	δ-PKC	Golgi
200mM Ethanol	δ-PKC	Perinucleus & Nucleus
50 nM D2 Dopamine receptor agonist NPA	δ-PKC	Perinucleus & Nucleus
10μM Dopamine receptor antagonist spiperone + 50 nM D2 Dopamine receptor agonist NPA	δ-PKC	Golgi
10μM Dopamine receptor antagonist spiperone + 200 mM ethanol	δ-PKC	Perinucleus & Nucleus
10 μM Adenosine receptor antagonist BW 1434 + 200 mM	δ-PKC	Perinucleus & Nucleus

Treatment	Protein	Predominant Localization
ethanol		
None	PKC Cα	Golgi
200mM Ethanol	PKC Cα	Nucleus & Cytoplasm
50 nM D2 Dopamine receptor agonist NPA	PKA Cα	Nucleus & Cytoplasm
10μM Dopamine receptor antagonist spiperone + 50 nM D2 Dopamine receptor agonist NPA	PKA Cα	Golgi
10μM Dopamine receptor antagonist spiperone + 200 mM ethanol	PKA Cα	Nucleus & Cytoplasm
10 μM Adenosine receptor antagonist BW 1434 + 200 mM ethanol (10 min exposure)	PKA Cα	Golgi
10 μM Adenosine receptor antagonist BW 1434 + 200 mM ethanol (48 hr exposure)	PKA Cα	Nucleus

Example 8: Mechanisms of Ethanol- and Dopamine-Mediated Translocation of PKC

The mechanisms underlying ethanol- and dopamine-mediated translocation of ε-PKC and δ-PKC were studied by altering the activity of various molecules that might be in the pathways leading to translocation.

When phospholipase C (PLC) activity was inhibited by the administration of pertussis toxin, both ethanol- and NPA-induced translocation of ε-PKC, δ-PKC and PKA Cα were inhibited in CHO and NG108-15 cells expressing the dopamine D2 receptor. Similarly, PLC inhibitors U-73122 and Et-18-OCH3 inhibited ethanol-induced translocation of ε-PKC by 87% in such CHO cells and by 84% and 96%, respectively, in such NG108-15 cells. This suggests that ethanol and dopamine cause translocation of ε-PKC and δ-PKC by activating PLC, which increases diacylglycerol levels.

Administration of PKA antagonist Rp-cAMPS blocked both ethanol- and NPA-induced translocation of ε PKC in CHO and NG108-12 cells expressing the dopamine D2 receptor but did not affect ethanol- or NPA-induced δ PKC translocation in such cells. Activation of PKA directly by administration of PKA agonist Sp-cAMPS or indirectly by using forskolin or PGE1 to increase cAMP levels

caused ε PKC to translocate to the cytoplasm, similar to ethanol- or NPA-induced translocation, but did not induce translocation of δ PKC. Thus, PKA activity is both necessary and sufficient to enable ethanol- and NPA-induced translocation of ε PKC to the cytoplasm but does not appear to have a role in ethanol- or NPA-induced translocation of δ PKC.

Without limitation to a particular theory, the inventors believe that the model shown in FIG. 17 explains the results presented in this Example 8 and the preceding Example 7. According to this model, dopamine/NPA induces PKA activation by releasing βγ, which activates adenylyl cyclase, and ethanol induces PKA activation by stimulating adenosine A2 receptors and by releasing βγ from G_i/G_o, which activate adenylyl cyclase. Thus, the ethanol- and dopamine- triggered pathways appear to intersect at adenylyl cyclase, which accounts for their shared translocation of PKA-Cα. These pathways also appear to intersect at PLC, which accounts for their shared, PKA-independent, translocation of δ PKC. The translocation of ε PKC as a result of NPA or ethanol exposure appears to be due to activation of PLC and PKA.

The inventors believe that δ-opioids and cannabinoids activate their respective receptors which in turn activate the same pathways that interact with ethanol-induced translocation of PKA-Cα, δ PKC and ε PKC in a manner that is analogous to the activation of the dopamine receptor described above.

Example 9: Synergistic Interactions of Ethanol and Other Addictive Drugs

The common signaling pathways of ethanol and dopamine D2 receptors demonstrated in Examples 7 and 8 was further explored in dosage studies. Although 30 minute incubations of dopamine D2-receptor-expressing NG108-15 and CHO cells with either 25mM ethanol or $5x10^{-10}$ M NPA did not cause any perceptible translocation of PKA-Cα, 30-minute co-incubation of these concentrations of ethanol and NPA caused maximal translocation of PKA-Cα. Translocation of ε PKC and δ PKC in such NG108-15 and CHO cells was induced by co-incubation with 10^{-9} M NPA and 25 mM ethanol, but neither agent alone caused translocation at these concentrations. When levels of CREB phosphorylation were measured using an antibody specific for phosphorylated CREB, co-incubation of 25mM ethanol and 1nM NPA for 10-180 minutes also showed synergistic interactions. These results demonstrate that ethanol and dopamine act synergistically with respect to translocation of PKA and PKC and CREB phosphorylation. They also suggest that

cells that express the dopamine D2 receptor are much more sensitive to low levels of ethanol than cells that do not express this receptor.

Synergistic translocation of PKA-Cα was also demonstrated after 10 minute co-incubations of: δ-opioid (10^{-11}M DADLE) and ethanol (25mM); cannabinoid ($2x10^{-10}$M methanindamide) and ethanol (25mM); δ-opioid (10^{-11}M DADLE) and dopamine (10^{-10}M NPA); and cannabinoid ($2x10^{-10}$M methanindamide) and dopamine (10^{-10}M NPA). However, a 10 minute coincubation of δ-opioid (10^{-11}M DADLE) and cannabinoid ($2x10^{-10}$M methanindamide) did not result in synergistic translocation of PKA-Cα.

Example 10: Imaging of GFP-tagged Fusion Proteins in Cells

The Green Fluorescent Protein (GFP) is employed as a tag to facilitate the imaging of certain proteins in mammalian tissue cell culture. For the current experiments we have constructed the GFP-tagged fusion proteins GFP-Cα and GFP-RIIβ. The fusion proteins PKCδ-GFP and PKCε-GFP were obtained from Christer Larsson, Lund University, Sweden. The methodology of the experiments is to introduce the DNA encoding these proteins into the cell of interest and utilize the cell's capacity to synthesize the foreign protein to image the movement of the protein under controlled conditions. Briefly, cDNA encoding the protein of interest were cloned into a pGFP vector (available from Clontech Laboratories, Inc.) under the control of the immediate early promoter of human cytomegalovirus (CMV). NG108-15 cells are cultured in a defined medium as described above, split onto plastic chamber slides, and allowed to establish for a further 24 hours. The cells are then transfected with 400ng of supercoiled plasmid in separate transfection experiments. The transfection is mediated by the commercially available Qiagen Effectene™ system. The efficiency of DNA transfer is about 30% of NG108-15 cells. These cells are allowed to express the protein for 20 hours prior to stimulation with the drug of interest. This reaction is terminated by washing and fixing the cells with 4% Paraformaldehyde.

Primary cell cultures of rat hippocampus include bipolar include bipolar and pyramidal neurons and glial cells. These cells are transfected with the same DNA concentration as above and with the same reagents, but the medium is maintained before and after transfection as these cells condition their own medium.

Imaging of the proteins is carried out on a BioRad laser scanning confocal microscope and a Leica upright microscope. These images are processed in Adobe Photoshop™ software and Spot™ software respectively.

The fusion constructs were made by linking, in frame, a polynucleotide encoding a 20Kd GFP and a polynucleotide encoding the protein of interest. Table 7 describes the primary structure of the resultant GFP-fusion proteins.

Table 7

GFP Fusion Proteins

Fusion	N-terminus	Spacer	C-terminus
GFP-PKA Cα	20kd GFP (aa 1-238)	his tag (20 aa)	~40kd PKA Cα (aa 1-351)
GFP-PKA RIIβ	20kd GFP (aa 1-238)	---	~52kd PKA RIIβ (aa 1-418)
δPKC-GFP	~76Kd δPKC (aa 1-676)	---	20kd GFP (aa 1-238)
εPKC-GFP	~94Kd εPKC (aa 1-737)	---	20kd GFP (aa 1-238)

Tables 8-10 indicate the translocation of several GFP fusion proteins. GFP-Cα translocated to the nucleus in ethanol exposed cells and cells exposed to NPA, as does PKA Cα. PKCδ-GFP translocated from the Golgi to the perinucleus, as does δ-PKC. GFP-RIIβ did not exhibit the translocation pattern observed for δ-PKC or RIIβ. This is likely due to overly high levels of expression, which may have saturated other cellular factors involved in the transaction. Lowering the expression levels should lead to the expected translocation pattern.

Table 8

Subcellular Localization of GFP-Cα

Cell Line	Control/Unstimulated	+100mM EtOH/30min	50nM NPA/30 min
NG108-15/D2R	Golgi Apparatus	Nucleus [>50% cells]	Nucleus [>50% cells]
1° Neurons	Golgi Apparatus	Nucleus [>50% cells]	ND*
1° Glia	Golgi Apparatus	Nucleus [<20% cells]	Nucleus [>50% cells]

Table 9

Subcellular Localization of PKCδ-GFP

Cell Line	Control/Unstimulated	+100mM EtOH/30min	50nM NPA/30 min
NG108-15/D2R	Golgi Apparatus	Perinucleus/membranes [>50% cells]	ND
1° Neurons	Golgi Apparatus	ND	ND
1° Glia	Golgi Apparatus	Golgi Apparatus	ND

Table 10

Subcellular Localization of GFP-RIIβ

Cell Line	Control/Unstimulated	+100mM EtOH/30min	50nM NPA/30 min
NG108-15/D2R	Golgi Apparatus	Golgi Apparatus	ND
1° Neurons	Golgi Apparatus	Golgi Apparatus	ND
1° Glia	Golgi Apparatus	Golgi Apparatus	ND

* = Not Determined

Example 11: Nuclear Localization of PKA RIIβ in Cells Exposed to Ethanol

NG-108 cells were exposed to 200mM ethanol for 48 hours. Whole cells homogenates and homogenates from isolated nuclei were subjected to SDS-PAGE. Western analysis was performed with antibodies which recognize PKA Cα, PKA Cβ, PKA RIIβ, and PKA RI. As shown in FIG. 13, PKA Cα and PKA RIIβ translocated to the nucleus and PKA Cβ and PKA RI did not translocate.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining prior exposure to ethanol of a test sample comprising at least one test cell, comprising:

(a) detecting in the test cell, the test cell being of a cell type in which a protein is present in a detectably different amount in a cellular subregion of a cell of the cell type which has been exposed to ethanol than in an unexposed cell of the cell type, the expression of a gene, the production of which is regulated by the protein, and

(b) comparing the amount of the gene expression thereby detected to the amount of the gene expression in a first control cell of a control sample which has not been exposed to ethanol or a second control cell which has been exposed to ethanol,

whereby a detectably different amount of the gene expression in test cell compared to the first control cell, or a detectably similar amount compared to the second control cell, is indicative of prior exposure to ethanol.

2. A method for determining prior exposure to an addictive drug other than ethanol of a test sample comprising at least one test cell, comprising:

(a) detecting in the test cell, the test cell being of a cell type in which a protein is present in a detectably different amount in a cellular subregion of a cell of the cell type which has been exposed to the addictive drug than in an unexposed cell of the cell type, the expression of a gene, the production of which is regulated by the protein, and

(b) comparing the amount of the gene expression thereby detected to the amount of the gene expression in a first control cell of a control sample which has not been exposed to the addictive drug or a second control cell which has been exposed to the addictive drug,

whereby a detectably different amount of the gene expression in test cell compared to the first control cell, or a detectably similar amount compared to the second control cell, is indicative of prior exposure to the addictive drug.

3. The method of Claim 1 or Claim 2, wherein the protein regulates expression of the gene through the phosphorylation of a factor.

4. The method of Claim 3, wherein the protein is PKA Cα.

5. The method of Claim 4, wherein the factor is a CREB.

6. A method for determining prior exposure to an addictive drug other than ethanol of a test sample comprising at least one test cell, comprising:

(a) detecting the presence or absence of a protein (P) in a first cellular subregion (S) of the test cell, the test cell being of a cell type (C) in which the protein is distributed differently between the first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus in an unexposed cell, and

(b) comparing the amount of protein thereby detected to the amount of the protein present in the first cellular subregion in a control cell of a control sample which has not been exposed to the addictive drug or a control cell which has been exposed to the addictive drug,

whereby a detectably different amount of the protein in the first cellular subregion of the test cell compared to the first control cell, or a detectably similar amount compared to the second control cell, is indicative of prior exposure to the addictive drug.

7. The method of Claim 6, wherein the test sample comprises a plurality of test cells and the control sample comprises a plurality of control cells, the method further comprising detecting a percentage of the test cells having a detectably different amount of the protein in the first cellular subregion, the percentage being a statistically significant greater percentage than a percentage of the control cells having a detectably different amount of the protein in the first cellular subregion.

8. The method of Claim 7, wherein the detecting comprises staining the test cells with a stain having specific binding affinity for the protein.

9. The method of Claim 7, wherein the detecting comprises detecting a change in the localization of color in said test cell relative to said first control cell.

10. The method of Claim 7, wherein the detectably different amount is a detectably greater amount.

11. The method of Claim 10, wherein the test cells are lymphocytes or neutrophils.

12. The method of Claim 11, wherein the test cells are lymphocytes.

13. The method of Claim 12, wherein the protein is PKA Cα and the first cellular subregion is the nucleus.

14. The method of Claim 10, wherein:

if P is PKA Cα and C is a lymphocyte or is derived from neural tissue, S is the nucleus;

if P is PKA RIα and C is a neutrophil or is derived from neural tissue, S is the nucleus;

if P is δ-PKC and C is derived from neural tissue, S is the perinucleus or the nucleus; and

if P is ∈-PKC and C is derived from neural tissue, S is the cytoplasm.

15. A method for determining prior chronic exposure to an addictive drug other than ethanol of a test sample comprising at least one test cell, comprising:

detecting the presence or absence of a protein in a first cellular subregion of the test cells, the test cells being of a cell type in which the protein is distributed differently between the first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus in an unexposed cell, and

comparing the amount of the protein thereby detected to the amount of the protein present in the first cellular subregion in a control cell of a control sample which has not been exposed to the addictive drug,

whereby a detectably different amount of the protein in the first cellular subregion of the test cell compared to the control cell is indicative of chronic exposure to the addictive drug.

16. The method of Claim 15, wherein the test sample comprises a plurality of test cells and the control sample comprises a plurality of control cells, the method further comprising detecting a percentage of the test cells having a detectably different amount of the protein in the first cellular subregion, the percentage being a statistically significant greater percentage than a percentage of the control cells having a detectably different amount of the protein in the first cellular subregion.

17. The method of Claim 16, wherein the sample is a blood sample.

18. The method of Claim 17, wherein the test cells are lymphocytes, the protein is PKA Cα, and the cellular subregion is the nucleus.

19. A method of determining whether a mammal has been chronically consuming an addictive drug other than ethanol, comprising:

obtaining a test sample from the mammal, the test sample comprising test cells of a cell type in which a protein is distributed differently between the first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus in an unexposed cell;

detecting the presence or absence of the protein in the first cellular subregion of the test cells; and

comparing the amount of the protein thereby detected to the amount of the protein present in the first cellular subregion in a control cell of a control sample which has not been exposed to the addictive drug,

whereby a detectably different amount of the protein in the first cellular subregion of the test cell compared to the control cell is indicative of the mammal having been chronically consuming the addictive drug.

20. The method of Claim 19, wherein the test sample comprises a plurality of test cells and the control sample comprises a plurality of control cells, the method further comprising detecting a percentage of the test cells having a detectably different amount of the protein in the first cellular subregion, the percentage being a statistically significant greater percentage than a percentage of the control cells having a detectably different amount of the protein in the first cellular subregion.

21. A method for identifying a substance that alters the effects of an addictive drug other than ethanol on the cellular localization of a protein in at least one test cell exposed to the addictive drug, comprising:

a. exposing a test sample containing the test cell to the substance, the test cell being of a cell type in which the protein is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus in an unexposed cell;

b. exposing the test cell to the addictive drug; and

c. detecting the presence or absence of the protein in the first cellular subregion,

whereby a detectably different amount of the protein in the first cellular subregion of the test cell compared to a control cell of a control sample which has been exposed to the addictive drug but not to the substance is indicative of the substance having altered the effects of the addictive drug on the cellular localization of the protein.

22. A method for identifying a substance that alters the effects of an addictive drug on the cellular localization of a protein, comprising:

a. exposing a test sample containing at least one test cell to the substance, the test cell being of a cell type in which the protein is distributed differently between an origin cellular subregion and a destination cellular subregion in a cell exposed to the addictive drug versus an unexposed cell;

b. exposing the test cell to a receptor agonist selected from the group consisting of an adenosine receptor agonist, a dopamine D2 receptor agonist, a δ-opiate receptor agonist, and a cannabinoid receptor agonist; and

c. detecting the presence or absence of the protein in the destination cellular subregion, whereby a detectably different amount of the protein in the destination cellular subregion of the test cell compared to a control cell of a control sample which has been exposed to the agonist but not to the substance is indicative of the substance having altered the effects of the addictive drug on the cellular localization of the protein.

23. The method of Claim 21, wherein the test sample comprises a plurality of test cells and the control sample comprises a plurality of control cells, the method further comprising detecting a percentage of the test cells having a detectably different amount of the protein in the first cellular subregion, the percentage being a statistically significant greater percentage than a percentage of the control cells having a detectably different amount of the protein in the first cellular subregion.

24. The method of Claim 23, wherein the first cellular subregion is a destination cellular subregion and said detectably different amount is a detectably greater amount.

25. The method of Claim 24, wherein the protein is present in a detectably greater amount in the destination cellular subregion of the addictive drug-exposed cells than in a second cellular subregion of the addictive drug-exposed cells.

26. The method of Claim 24, wherein the substance is an inhibitor of the effects of the addictive drug on the cellular localization of the protein if the amount of protein in the destination cellular subregion of the test cells is a detectably lesser amount than in the control cells.

27. The method of Claim 24, wherein the substance is an enhancer of the effects of the addictive drug on the cellular location of the protein if the amount of protein in the destination cellular subregion of the test cells is a detectably greater amount than in the control cells.

28. The method of Claim 24, wherein the substance is an inhibitor of the effects of the addictive drug on the cellular localization if the protein is present in the destination cellular subregion in a detectably lesser percentage of the test cells than in the control cells.

29. The method of Claim 24, wherein the substance is an enhancer of the effects of the addictive drug on the cellular localization if the protein is present in the destination cellular subregion in a detectably greater percentage of the test cells than in the control cells.

30. The method of Claim 23, wherein the first cellular subregion is an origin cellular subregion and said detectably different amount is a detectably lesser amount.

31. The method of Claim 30, wherein the substance is an inhibitor of the effects of the addictive drug on the cellular location of the protein if the amount of protein in the origin cellular subregion of the test cells is a detectably greater amount than in the control cells.

32. The method of Claim 30, wherein the protein is present in a detectably lesser amount in the origin cellular subregion than in a second cellular subregion of the addictive drug-exposed cells.

33. The method of Claim 30, wherein the substance is an enhancer of the effects of the addictive drug on the cellular location of the protein if the amount of protein in the origin cellular subregion of the test cells is a detectably lesser amount than in the control cells.

34. The method of Claim 30, wherein the substance is an inhibitor of the effects of the addictive drug on the cellular localization if the protein is present in the origin cellular subregion in a detectably greater percentage of the test cells than in the control cells.

35. The method of Claim 30, wherein the substance is an enhancer of the effects of the addictive drug on the cellular localization if the protein is present in the origin cellular subregion in a detectably lesser number of the test cells than in the control cells.

36. The method of Claim 30, wherein the protein is not detectable in the addictive drug-exposed cell.

38. The method of Claim 30, wherein the cell type is a Purkinje cell.

39. The method of Claim 23, wherein the protein is selected from the group consisting of PKA Cα, PKA-RIα, PKA-RIIβ, α-PKC, δ-PKC and ∈-PKC.

40. The method of Claim 23, wherein the protein is a PKA subunit.

41. The method of Claim 40, wherein the PKA subunit is PKA Cα.

42. The method of Claim 40, wherein the PKA subunit is PKA RIα.

43. The method of Claim 40, wherein the PKA subunit is PKA RIIβ.

44. The method of Claim 23, wherein the protein is δ-PKC.

45. The method of Claim 23, wherein the protein is ε-PKC.

46. The method of Claim 23, wherein the cell type is, or is derived from, cells selected from the group consisting of neural cells, fibroblast cells, lymphocytes, granulocytes and CHO cells.

47. The method of Claim 23, wherein the cell type is a primary cell.

48. The method of Claim 23, wherein the cell type is a cultured cell line.

49. The method of Claim 23, wherein step (c) comprises detecting a colorimetric change.

50. The method of Claim 49, wherein step (c) further comprises observing the subcellular distribution of a stain specific for the protein.

51. The method of Claim 49, wherein prior to the addictive drug exposure, the first cellular subregion is labeled with a first substance and the protein is labeled with a second substance, wherein the first and second substances produce the colorimetric change when combined.

52. The method of Claim 30, wherein the first cellular subregion is the Golgi.

53. The method of Claim 52, wherein step (c) comprises detecting the expression of a gene regulated by a factor which is phosphorylated by the protein.

54. The method of Claim 53, wherein the gene is a CREB regulated gene.

55. The method of Claim 54, wherein the gene is a reporter gene.

56. The method of Claim 55, wherein the protein is PKA Cα.

57. The method of Claim 55, wherein the protein is δ-PKC.

58. The method of Claim 24, wherein the destination cellular subregion is the cytoplasm.

59. The method of Claim 23, wherein the test cells are exposed to the substance before the addictive drug.

60. The method of Claim 21, wherein said substance is selected from the group consisting of:

adenosine A1 receptor agonists;
adenosine A1 receptor antagonists;
adenosine A2 receptor agonists;
adenosine A2 receptor antagonists;
dopamine D2 receptor agonists;
dopamine D2 receptor antagonists;
δ-opiate receptor agonists;
δ-opiate receptor antagonists;
cannabinoid receptor agonists;
cannabinoid receptor antagonists;
PKA receptor agonists;

PKA receptor antagonists;
phospholipase C receptor agonists;
phospholipase C receptor antagonists;
activators of adenylylcyclase;
inhibitors of adenylylcyclase;
activators of G_i, G_o and G_z mediated functions;
inhibitors of G_i, G_o and G_z mediated functions; and
structurally related compounds.

61. A method for identifying a substance that mimics the effects of ethanol on the cellular location of a protein in at least one test cell, comprising:

(a) exposing a sample comprising the test cell to the substance, but not to ethanol, the test cell being of a cell type in which the protein is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to ethanol than in an unexposed cell; and

(b) detecting the presence or absence of the protein in the first cellular subregion of the test cell;

whereby detection of a similar amount of the protein in the first cellular subregion of the test cell compared to a first control cell which has been exposed to ethanol but not to the substance is indicative of the substance having mimicked the effects of ethanol on the cellular location of the protein.

62. A method for identifying a substance that mimics the effects of an addictive drug other than ethanol on the cellular location of a protein in at least one test cell, comprising:

(a) exposing a sample comprising the test cell to the substance, but not to the addictive drug, the test cell being of a cell type in which the protein is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug than in an unexposed cell; and

(b) detecting the presence or absence of the protein in the first cellular subregion of the test cell;

whereby detection of a similar amount of the protein in the first cellular subregion of the test cell compared to a first control cell which has been exposed to

the addictive drug but not to the substance is indicative of the substance having mimicked the effects of the addictive drug on the cellular location of the protein.

63. A method for identifying a substance that mimics the effects of an addictive drug on the cellular localization of a protein, comprising:

a. exposing a test sample containing at least one test cell to the substance, the test cell being of a cell type in which the protein is distributed differently between an origin cellular subregion and a destination cellular subregion in a cell exposed to the addictive drug versus an unexposed cell; and

b. detecting the presence or absence of the protein in the destination cellular subregion of the test cell,

whereby a detectably similar amount of the protein in the destination cellular subregion of the test cell compared to a control cell of a control sample which has been exposed to a receptor agonist selected from the group consisting of: an adenosine receptor agonist, a dopamine D2 receptor agonist, a δ-opiate receptor agonist, and a cannabinoid receptor agonist, but not exposed to the substance, is indicative of the substance having mimicked the effects of the addictive drug on the cellular localization of the protein.

64. The method of Claim 61 or 62, wherein said substance is selected from the group consisting of:

adenosine A1 receptor agonists;
adenosine A2 receptor agonists;
dopamine D2 receptor agonists;
δ-opiate receptor agonists;
cannabinoid receptor agonists;
PKA receptor agonists;
phospholipase C receptor agonists;
activators of adenylcyclase;
activators of G_i, G_o and G_z mediated functions; and
structurally related compounds.

65. A method for identifying a substance that modulates the cellular effects of an addictive drug, comprising:

a. exposing a test sample containing at least one test cell to the addictive drug, the test cell being of a cell type in which a detectably greater amount of a target protein is phosphorylated in a cell exposed to the addictive drug than in a cell not exposed to the addictive drug;

b. exposing the test cell to the substance; and

c. detecting the presence or absence of the phosphorylated target protein,

whereby a detectably different amount of the phosphorylated target protein in the test cell compared to a control cell of a control sample which has been exposed to the addictive drug but not to the substance is indicative of the substance having modulated the cellular effects of the addictive drug.

66. The method of Claim 65, wherein said detecting comprises exposing the test cell or an extract from the test cell to a phosphoantibody.

67. The method of Claim 65, wherein the target protein is phosphorylated by a protein that is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus in an unexposed cell.

68. The method of Claim 67, wherein the indicative protein is PKA Cα.

69. The method of Claim 65, wherein the target protein is CREB.

70. A method for identifying a substance that modulates the cellular effects of an addictive drug, comprising:

a. exposing a test sample containing at least one test cell to the addictive drug, the test cell being of a cell type in which detectably greater expression of a gene occurs in a cell exposed to the addictive drug than in a cell not exposed to the addictive drug;

b. exposing the test cell to the substance; and

c. detecting the gene expression,

whereby a detectably different amount of the gene expression in the test cell compared to a control cell of a control sample which has been exposed to the addictive drug but not to the substance is indicative of the substance having modulated the cellular effects of the addictive drug.

71. The method of Claim 70, wherein the gene expression is regulated by a factor which is phosphorylated in a cell exposed to the addictive drug.

72. The method of Claim 71, wherein the factor is CREB.

73. The method of Claim 72, wherein the gene is a reporter gene.

74. A kit for use in determining prior cellular exposure to an addictive drug other than ethanol of a sample comprising at least one test cell, comprising:

a stain specific for a protein which is distributed differently between a first cellular subregion and a second cellular subregion in a cell of the same cell type as said test cell which has been exposed to the addictive drug than in an unexposed cell, and

instructions for comparing the localization of said stain in said test cell to the localization of said stain in a first control cell which has been exposed to the addictive drug or a second control cell which has not been exposed to the addictive drug, said first and second control cells being of the same cell type as said test cell.

75. The kit of Claim 74, further comprising at least one reagent required for the detection of a complex between the protein and the stain.

76. The kit of Claim 74, further comprising a reference standard.

77. The kit of Claim 74, further comprising printed instructions for detecting the amount of an ethanol indicative protein in a first cellular subregion of said test cell.

78. The kit of Claim 74, wherein the ethanol indicative protein is selected from the group consisting of PKA Cα, PKA RIα, PKA RIIβ, δ-PKC, and ∈-PKC.

79. A kit for detecting, in a test sample derived from at least one test cell, prior exposure of said test cell to ethanol, comprising:

a stain specific for a gene product, or polynucleotide encoding said gene product, the production of which is regulated by a protein which is present in a detectably different amount in a first cellular subregion in a cell which has been exposed to ethanol than in an unexposed cell, and

instructions for comparing the amount of said stain detected in said sample to the amount of said stain in a control sample derived from a first control cell which has been exposed to ethanol or a second control cell which has not been exposed to ethanol, said first and second control cells being of the same cell type as said control cell.

80. A kit for detecting, in a test sample derived from at least one test cell, prior exposure of said test cell to an addictive drug other than ethanol, comprising:

a stain specific for a gene product, or polynucleotide encoding said gene product, the production of which is regulated by a protein which is present in a detectably different amount in a first cellular subregion in a cell which has been exposed to the addictive drug than in an unexposed cell, and

instructions for comparing the amount of said stain detected in said sample to the amount of said stain in a control sample derived from a first control cell which has been exposed to the addictive drug or a second control cell which has not been exposed to the addictive drug, said first and second control cells being of the same cell type as said control cell.

81. A kit for identifying a substance that alters or mimics the effects of ethanol on the cellular localization of a protein, comprising:

a stain specific for a gene product, or a polynucleotide encoding said gene product, the production of which is regulated by a protein which is present in a detectably different amount in a first cellular subregion in a cell which has been exposed to ethanol than in an unexposed cell; and

instructions for comparing:

(1) the amount of said stain detected in a sample derived from a test cell, said test cell having been exposed to the substance and ethanol where the

substance is tested for its ability to alter the effects of ethanol, and has been exposed the substance, but not to ethanol, where the substance is tested for its ability to mimic the effects of ethanol; to

(2) the amount of said stain in a control sample derived from a first control cell which has been exposed to ethanol or a second control cell which has not been exposed to ethanol, said first and second control cells being of the same cell type as said control cell.

82. A kit for identifying a substance that alters or mimics the effects of an addictive drug other than ethanol on the cellular localization of a protein, comprising:

a stain specific for a gene product, or a polynucleotide encoding said gene product, the production of which is regulated by a protein which is present in a detectably different amount in a first cellular subregion in a cell which has been exposed to the addictive drug than in an unexposed cell, and

instructions for comparing:

(1) the amount of said stain detected in a sample derived from a test cell, said test cell having been exposed to the substance and ethanol where the substance is tested for its ability to alter the effects of the addictive drug, and has been exposed the substance, but not to the addictive drug, where the substance is tested for its ability to mimic the effects of ethanol; to

(2) the amount of said stain in a control sample derived from a first control cell which has been exposed to the addictive drug or a second control cell which has not been exposed to the addictive drug, said first and second control cells being of the same cell type as said control cell.

83. The kit of Claim 81 or 82 further comprising polynucleotide comprising a reporter gene.

84. The kit of Claim 83 further comprising a host cell transfected with said polynucleotide.

85. A kit for identifying a substance that alters or mimics the effects of an addictive drug other than ethanol on the cellular localization of a protein, comprising:

a stain specific for a protein which is distributed differently between a first cellular subregion and a second cellular subregion in a cell of the same cell type as a test cell which has been exposed to the addictive drug than in an unexposed cell, and

printed instructions for comparing the localization of said stain in a test cell to the localization of said stain in a first control cell which has been exposed to the addictive drug or a second control cell which has not been exposed to the addictive drug, said first and second control cells being of the same cell type as said test cell.

86. The kit of Claim 85, comprising instructions for comparing the localization of said stain in said test cell to the localization of said stain in both said first and said second control cells.

87. A kit for identifying a substance that alters or mimics the effects of ethanol on the cellular localization of a protein, comprising:

a stain specific for a phosphorylated form of a factor which is phosphorylated by a protein which is present in a detectably different amount in a first cellular subregion in a cell which has been exposed to ethanol than in an unexposed cell; and

instructions for comparing:

(1) the amount of said stain detected in a sample derived from a test cell, said test cell having been exposed to the substance and ethanol where the substance is tested for its ability to alter the effects of ethanol, or said test cell having been exposed the substance, but not to ethanol, where the substance is tested for its ability to mimic the effects of ethanol; to

(2) the amount of said stain in a control sample derived from a first control cell which has been exposed to ethanol or a second control cell which has not been exposed to ethanol, said first and second control cells being of the same cell type as said test cell.

88. A kit for identifying a substance that alters or mimics the effects of an addictive drug other than ethanol on the cellular localization of a protein, comprising:

a stain specific for a phosphorylated form of a factor which is phosphorylated by a protein which is present in a detectably different amount in a first cellular subregion in a cell which has been exposed to the addictive drug than in an unexposed cell; and

instructions for comparing:

(1) the amount of said stain detected in a sample derived from a test cell, said test cell having been exposed to the substance and the addictive drug where the substance is tested for its ability to alter the effects of the addictive drug, or said test cell having been exposed the substance, but not to the addictive drug, where the substance is tested for its ability to mimic the effects of the addictive drug; to

(2) the amount of said stain in a control sample derived from a first control cell which has been exposed to the addictive drug or a second control cell which has not been exposed to the addictive drug, said first and second control cells being of the same cell type as said test cell.

89. The use of a substance that mimics or enhances the effects of an addictive drug on the subcellular localization of a protein which is distributed differently among the cellular subregions of a cell which has been exposed to the addictive drug compared to a cell of the same cell type which has not been exposed to the addictive drug, in the preparation of a medicament for reducing consumption of the addictive drug..

90. The use of Claim 89, wherein said addictive drug is ethanol.

91. The use of Claim 90, wherein said substance is selected from the group consisting of an adenosine receptor agonist, an opioid receptor agonist, and a cannabinoid receptor agonist.

92. The use of Claim 89, wherein said addictive drug is an opioid.

93. The use of Claim 92, wherein said substance is an adenosine receptor agonist.

94. The use of Claim 89, wherein said addictive drug is a cannabinoid.

95. The use of Claim 94, wherein said substance is selected from the group consisting of an adenosine receptor agonist and a dopamine receptor agonist.

96. The use of a substance that inhibits the effects of an addictive drug on the subcellular localization of a protein which is distributed differently among the cellular subregions of a cell which has been exposed to said addictive drug compared to a cell of the same cell type which has not been exposed to said addictive drug, in the preparation of a medicament for reducing consumption of the addictive drug.

97. The use of Claim 96, wherein said addictive drug is ethanol.

98. The use of Claim 97, wherein said substance is selected from the group consisting of an adenosine receptor antagonist, an opioid receptor antagonist, and a cannabinoid receptor antagonist.

99. The use of Claim 96, wherein said addictive drug is an opioid.

100. The use of Claim 99, wherein said substance is an adenosine receptor antagonist.

101. The use of Claim 96, wherein said addictive drug is a cannabinoid.

102. The use of Claim 101, wherein said substance is selected from the group consisting of an adenosine receptor antagonist and a dopamine receptor antagonist.

103. A use of a first substance and a second substance in the preparation of a medicament for reducing consumption of an addictive drug, wherein the first substance inhibits the activity of a receptor for the addictive drug and the second substance inhibits the activity of a factor that acts in synergy with the receptor to alter the subcellular localization of a protein which is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus an unexposed cell.

104. The use of Claim 103, wherein the addictive drug is ethanol.

105. The use of Claim 104, wherein the first substance is an adenosine receptor antagonist.

106. The use of Claim 105, wherein the second substance is selected from the group consisting of: a δ-opioid receptor antagonist, a dopamine receptor antagonist and a cannibinoid receptor antagonist.

107. The use of Claim 103, wherein the addictive drug is a δ-opioid.

108. The use of Claim 107, wherein the first substance is a δ-opioid receptor antagonist.

109. The use of Claim 108, wherein the second substance is selected from the group consisting of: an adenosine receptor antagonist and a dopamine receptor antagonist.

110. The use of Claim 103, wherein the addictive drug is a cannibinoid.

111. The use of Claim 110, wherein the first substance is a cannibinoid receptor antagonist.

112. The use of Claim 111, wherein the second substance is selected from the group consisting of: an adenosine receptor antagonist and a dopamine receptor antagonist.

113. A use of a dopamine receptor antagonist and a substance in the preparation of a medicament for reducing consumption of an addictive drug, wherein the addictive drug causes an increase in dopamine levels and the substance inhibits the activity of a factor that acts in synergy with a dopamine receptor to alter the subcellular localization of a protein which is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus an unexposed cell.

114. The use of Claim 113, wherein the substance is selected from the group consisting of: an adenosine receptor antagonist, a δ-opioid receptor antagonist and a cannibinoid receptor antagonist.

115. A use of a first substance and a second substance in the preparation of a medicament for reducing consumption of an addictive drug, wherein the first substance enhances the activity of a receptor for the addictive drug and the second substance enhances the activity of a factor that acts in synergy with the receptor to alter the subcellular localization of a protein which is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus an unexposed cell.

116. The use of Claim 115, wherein the addictive drug is ethanol.

117. The use of Claim 116, wherein the first substance is an adenosine receptor agonist.

118. The use of Claim 117, wherein the second substance is selected from the group consisting of: a δ-opioid receptor agonist, a dopamine receptor agonist and a cannibinoid receptor agonist.

119. The use of Claim 115, wherein the addictive drug is a δ-opioid.

120. The use of Claim 119, wherein the first substance is a δ-opioid receptor agonist.

121. The use of Claim 120, wherein the second substance is selected from the group consisting of: an adenosine receptor agonist and a dopamine receptor agonist.

122. The use of Claim 115, wherein the addictive drug is a cannibinoid.

123. The use of Claim 122, wherein the first substance is a cannibinoid receptor agonist.

124. The use of Claim 123, wherein the second substance is selected from the group consisting of: an adenosine receptor agonist and a dopamine receptor agonist.

125. A use of a dopamine receptor agonist and a substance in the preparation of a medicament for reducing consumption of an addictive drug, wherein the addictive drug causes an increase in dopamine levels and the substance enhances the activity of a factor that acts in synergy with a dopamine receptor to alter the subcellular localization of a protein which is distributed differently between a first cellular subregion and a second cellular subregion in a cell exposed to the addictive drug versus an unexposed cell.

126. The use of Claim 125, wherein the substance is selected from the group consisting of: an adenosine receptor antagonist, a δ-opioid receptor agonist and a cannibinoid receptor agonist.

127. A pharmaceutical composition comprising a substance identified by the method of Claim 61 formulated to comprise, in unit dosage form, an amount effective to reduce consumption of ethanol.

128. A pharmaceutical composition comprising a substance identified by the method of Claims 21 or 62 formulated to comprise, in unit dosage form, an amount effective to reduce consumption of an addictive drug other than ethanol.

FIG. 1A

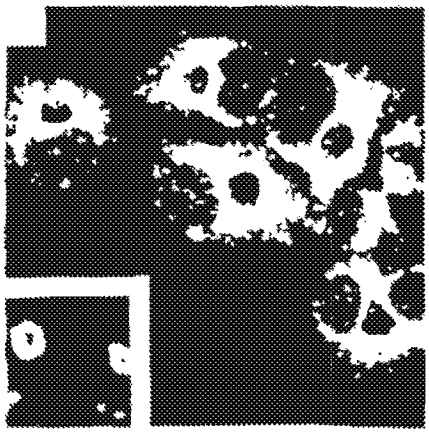
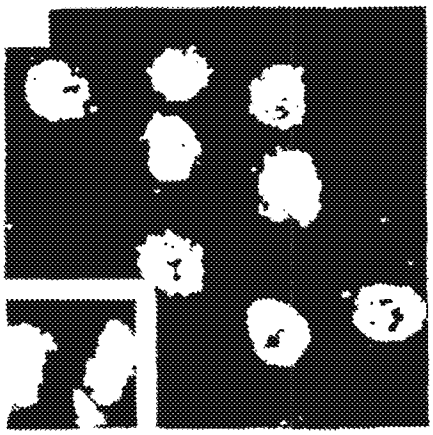


FIG. 1B



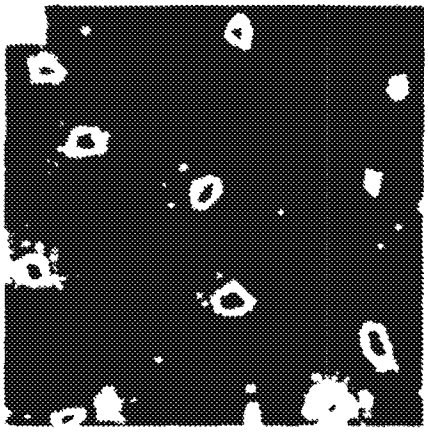


FIG. 1C

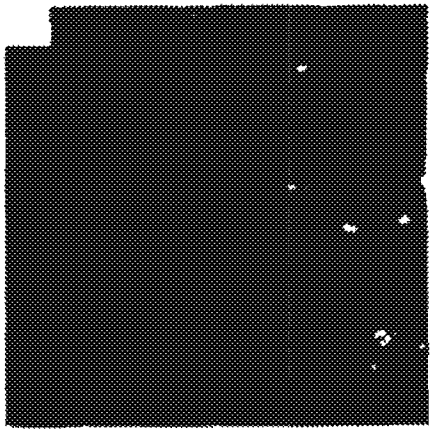


FIG. 1D

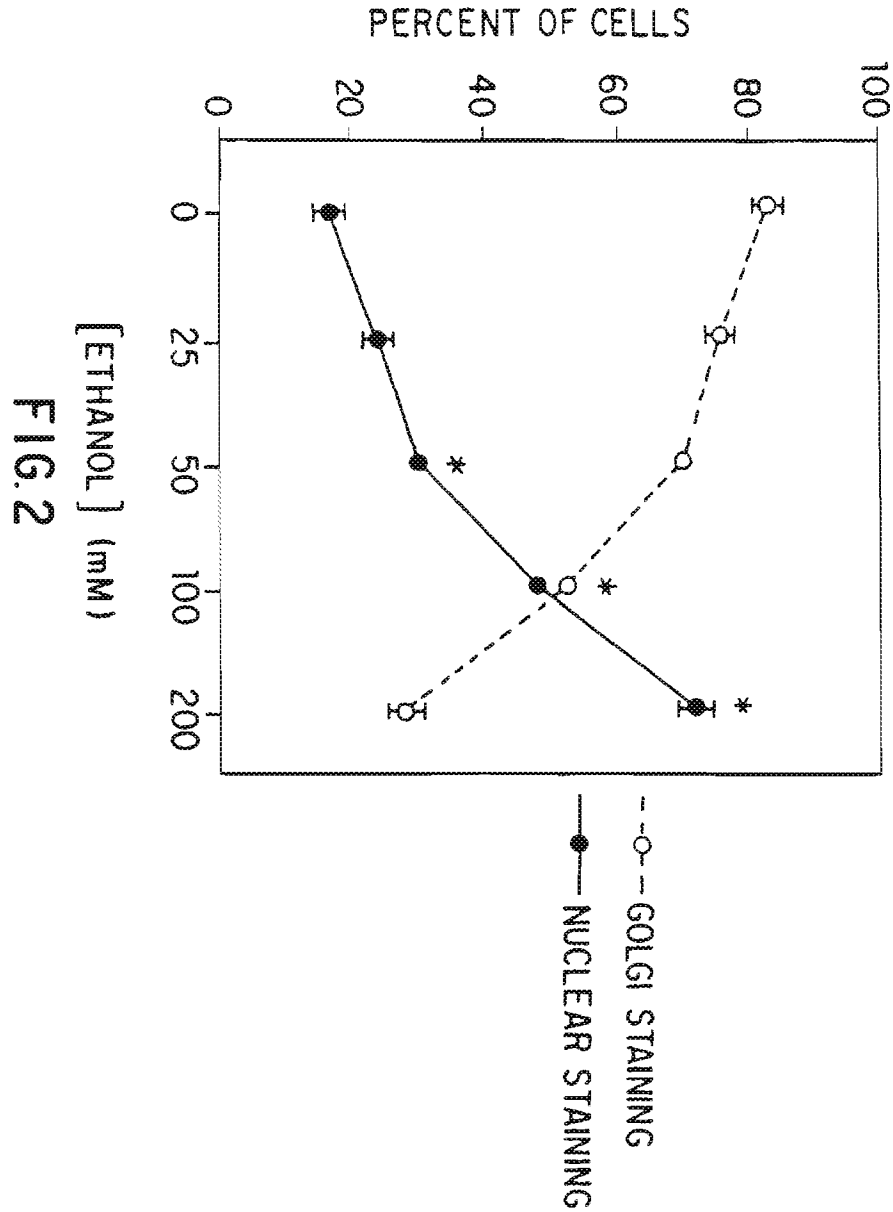

PERCENT OF CELLS
0
20
40
60
80
100
0
25
50
100
200
[ETHANOL] (mM)
NUCLEAR STAINING
GOLGI STAINING

FIG. 2

CONTROL

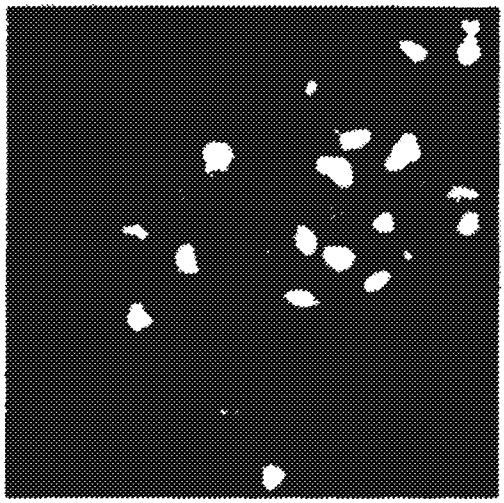


FIG.3A

ETHANOL

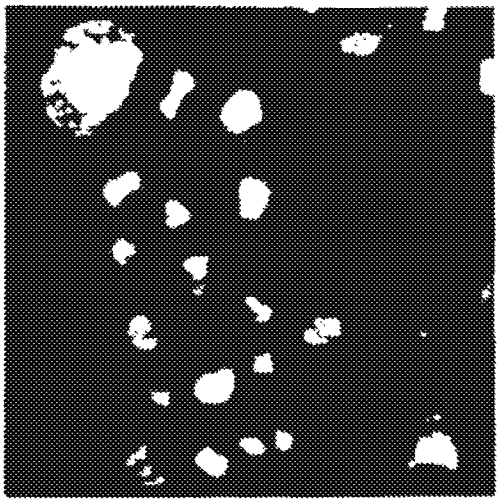


FIG.3B

PGE_1

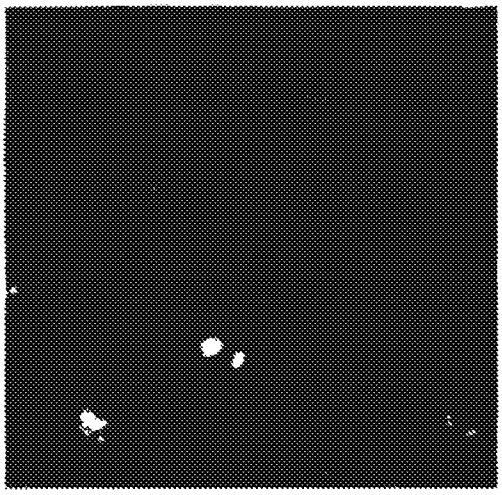


FIG.3C

FORSKOLIN

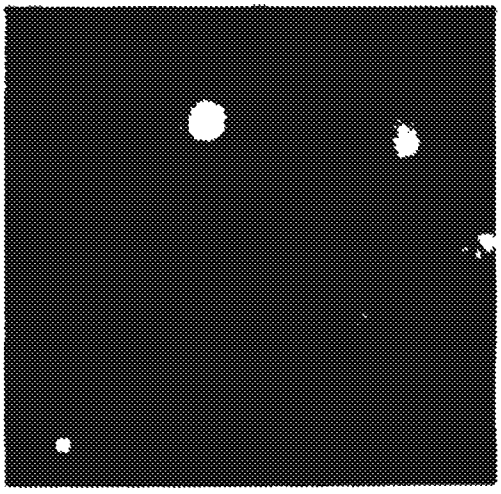


FIG.3D

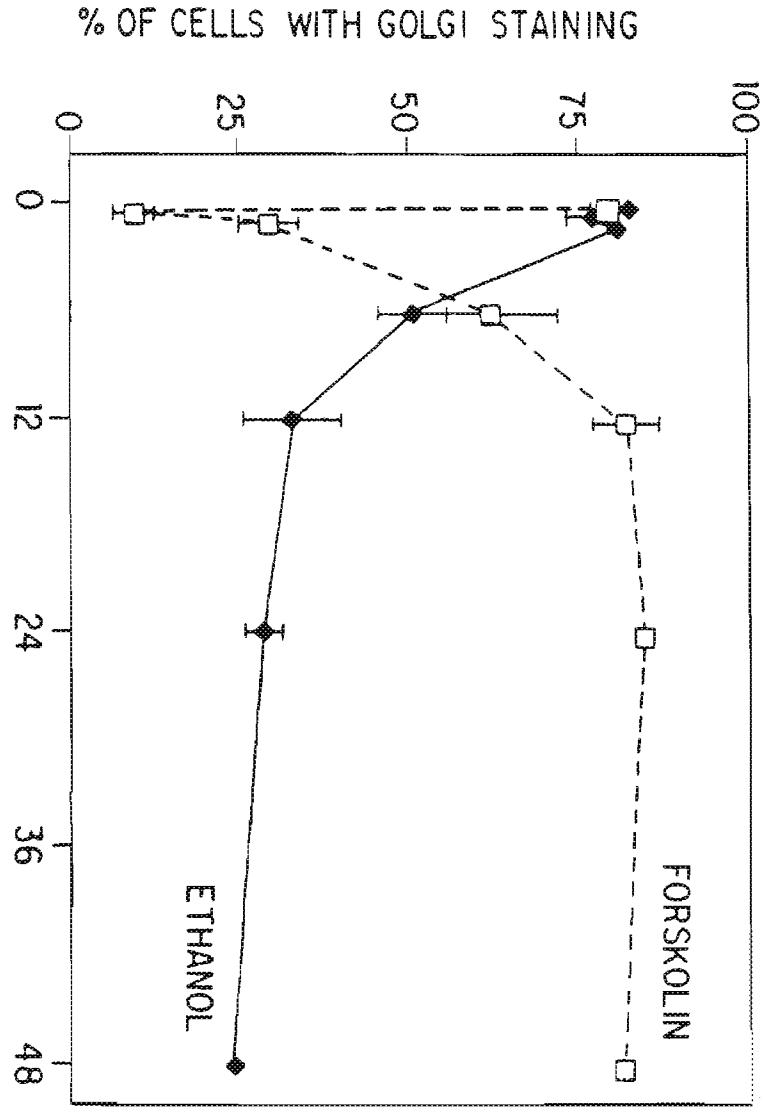

% OF CELLS WITH GOLGI STAINING
0
25
50
75
100
0
12
24
36
48
TIME (Hrs.)
ETHANOL
FORSKOLIN

FIG. 4

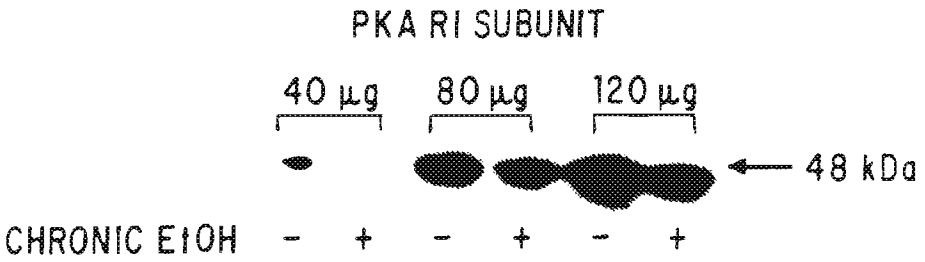

PKA RI SUBUNIT
40 µg
80 µg
120 µg
48 kDa
CHRONIC EtOH - + - + - +

FIG.5A

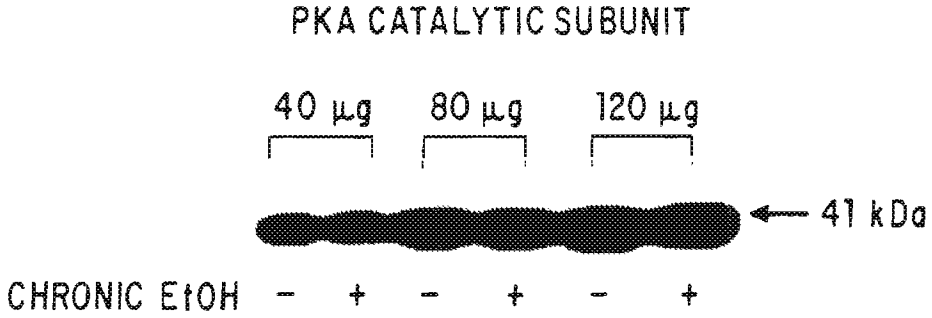

PKA CATALYTIC SUBUNIT
40 µg
80 µg
120 µg
41 kDa
CHRONIC EtOH - + - + - +

FIG.5B

PKC δ

CONTROL

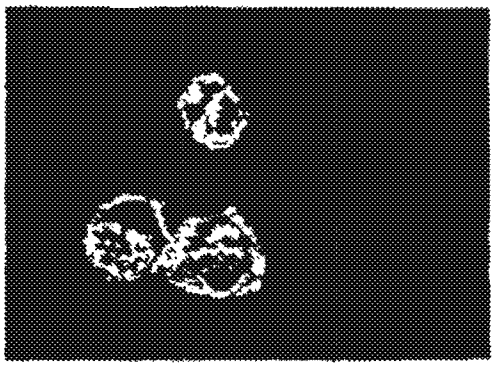


FIG.6A

EtOH
200 mM

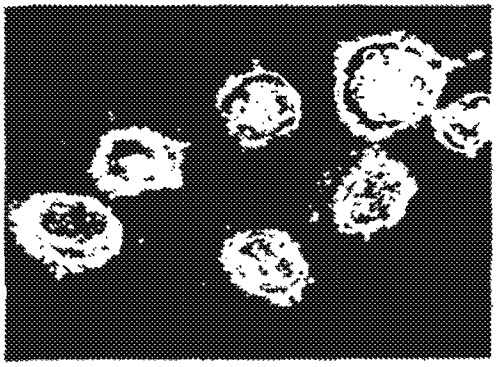


FIG.6B

PMA

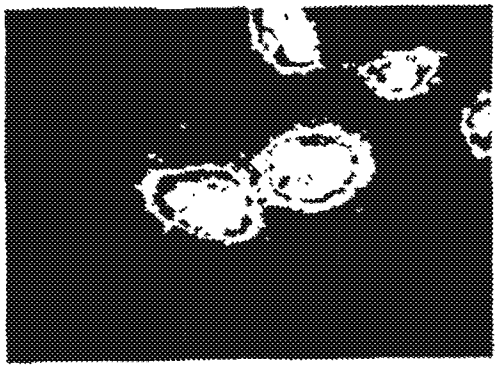


FIG.6C

PKC δ

CONTROL

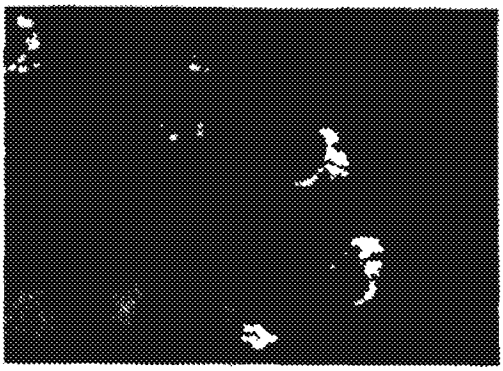


FIG. 7A

EtOH
25 mM

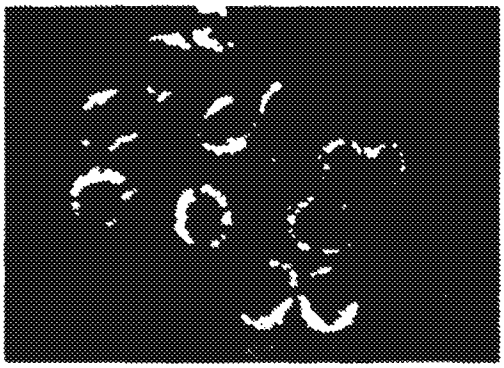


FIG. 7B

Pre.

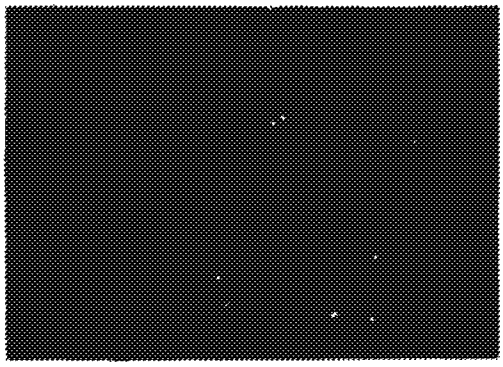


FIG. 7C

PKC ε

CONTROL

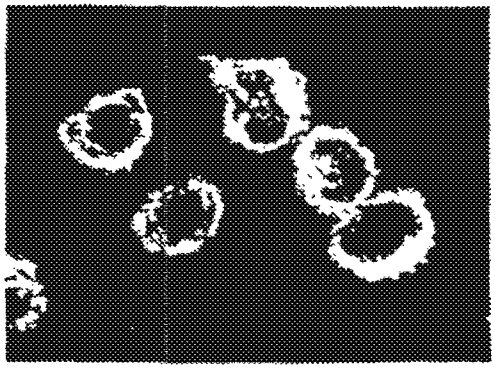


FIG.8A

EtOH
200 mM

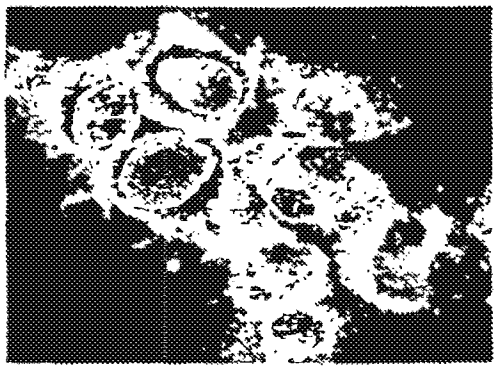


FIG.8B

PMA

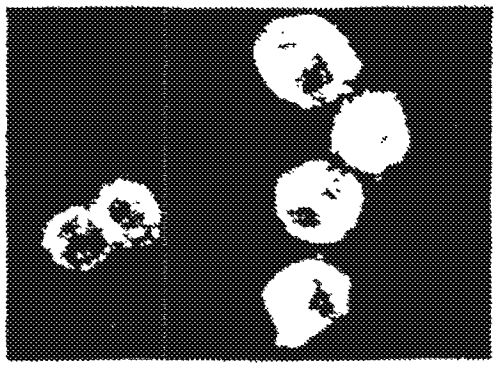


FIG.8C

PKC ε

CONTROL

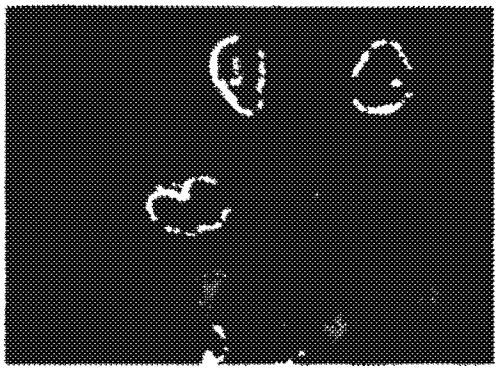


FIG.9A

EtOH
25 mM

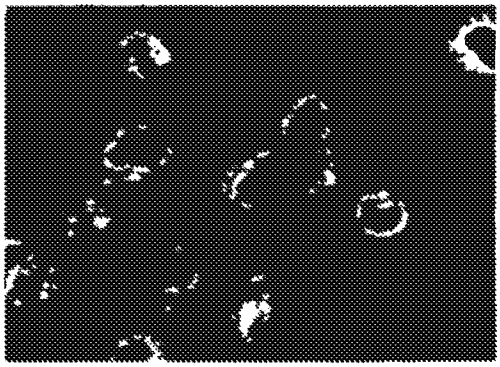


FIG.9B

Pre.

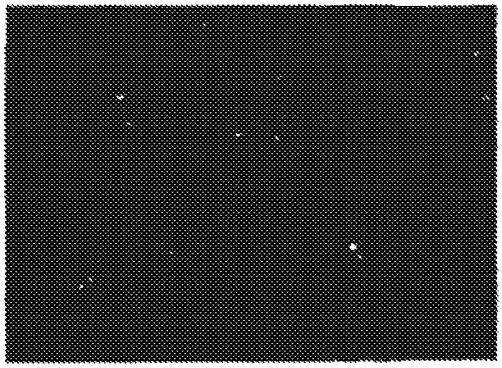
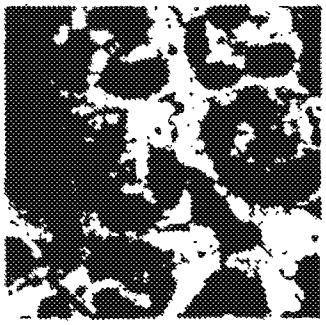
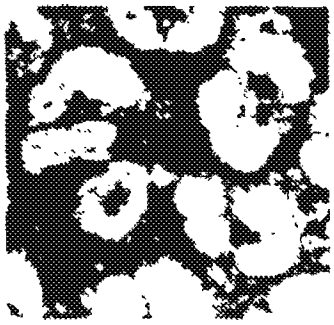


FIG.9C



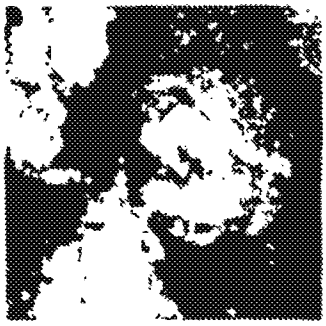
Control

FIG.10A



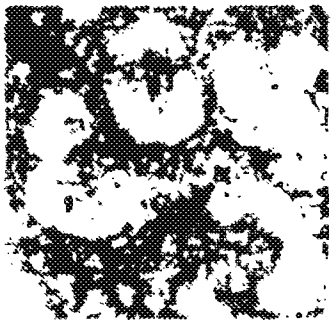
Alcoholic

FIG.10B



Control + EtOH

FIG.10C



Alcoholic + EtOH

FIG.10D

Normal control
PKA Cat. α mainly cytoplasm

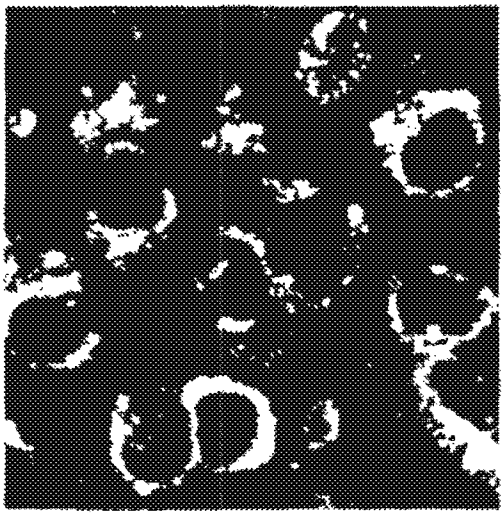


FIG.11A

Alcoholic
PKA Cat. α is mainly in the nucleus

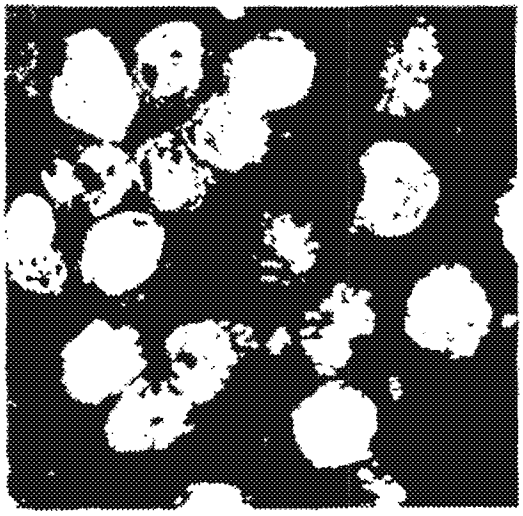


FIG.11B

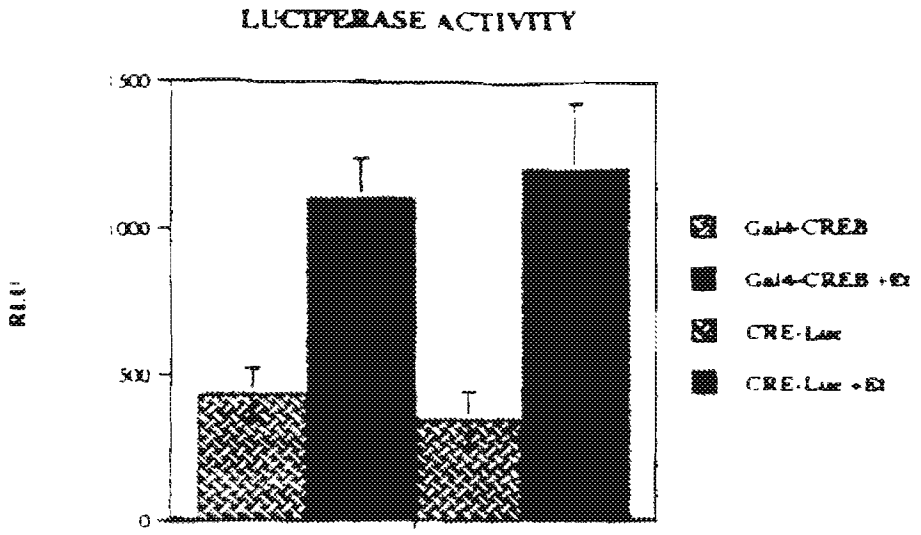


FIG. 12

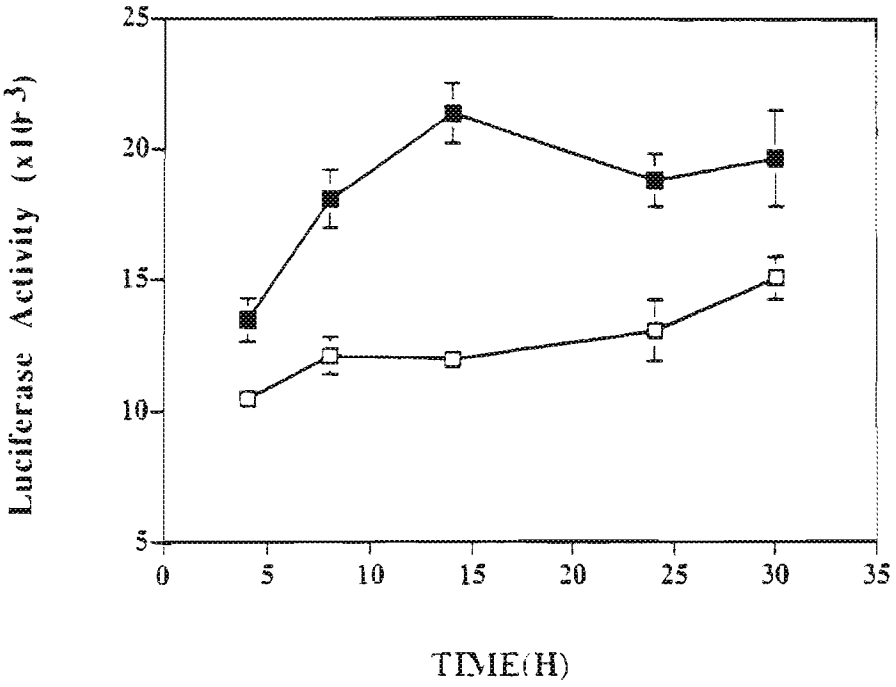

Luciferase Activity (x10E-3)
25
20
15
10
5
0
5
10
15
20
25
30
35
TIME(H)

FIG. 13

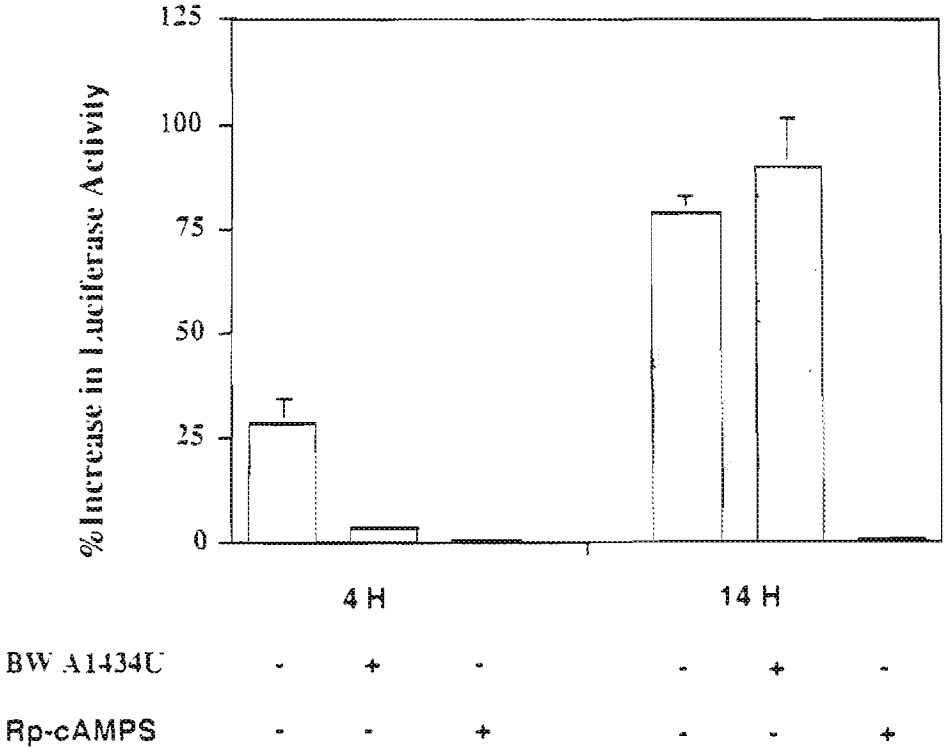

125
100
75
50
25
0
% Increase in Luciferase Activity
4 H
14 H
BW A1434U - + - - + -
Rp-cAMPS - - + - - +

FIG. 14

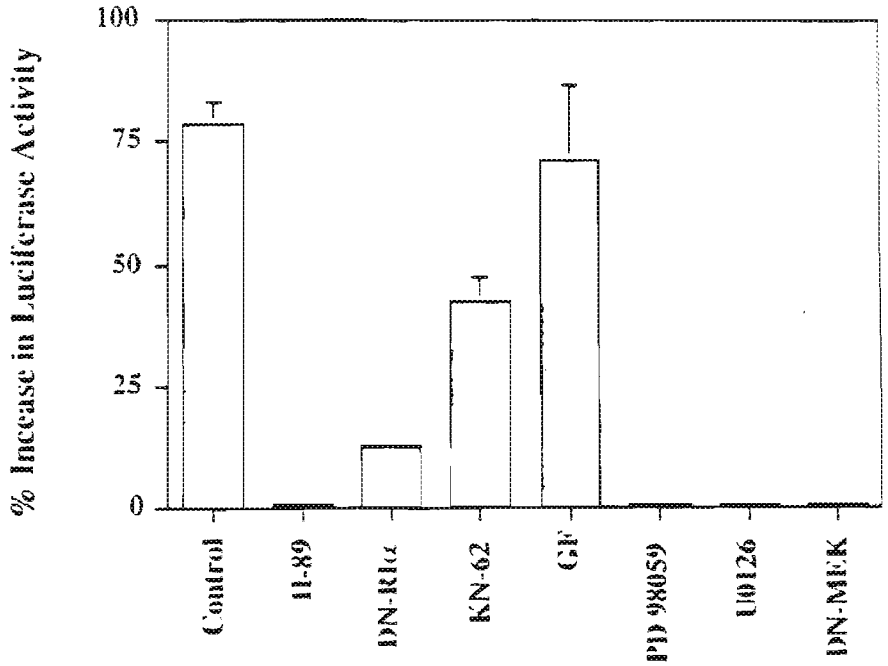

% Increase in Luciferase Activity
100
75
50
25
0
Control
BN-Ras
KN-62
PD 98059
U0126
DN-MEK

FIG. 15

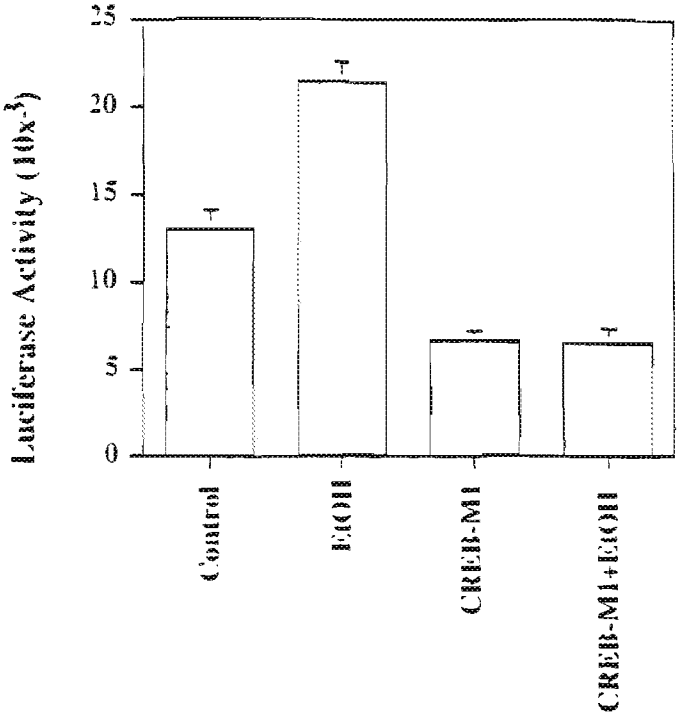


FIG. 16A

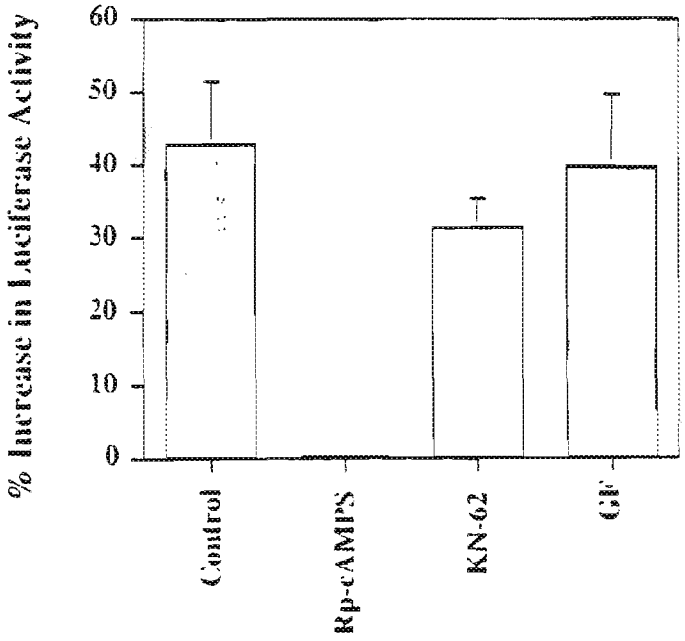


FIG. 16B

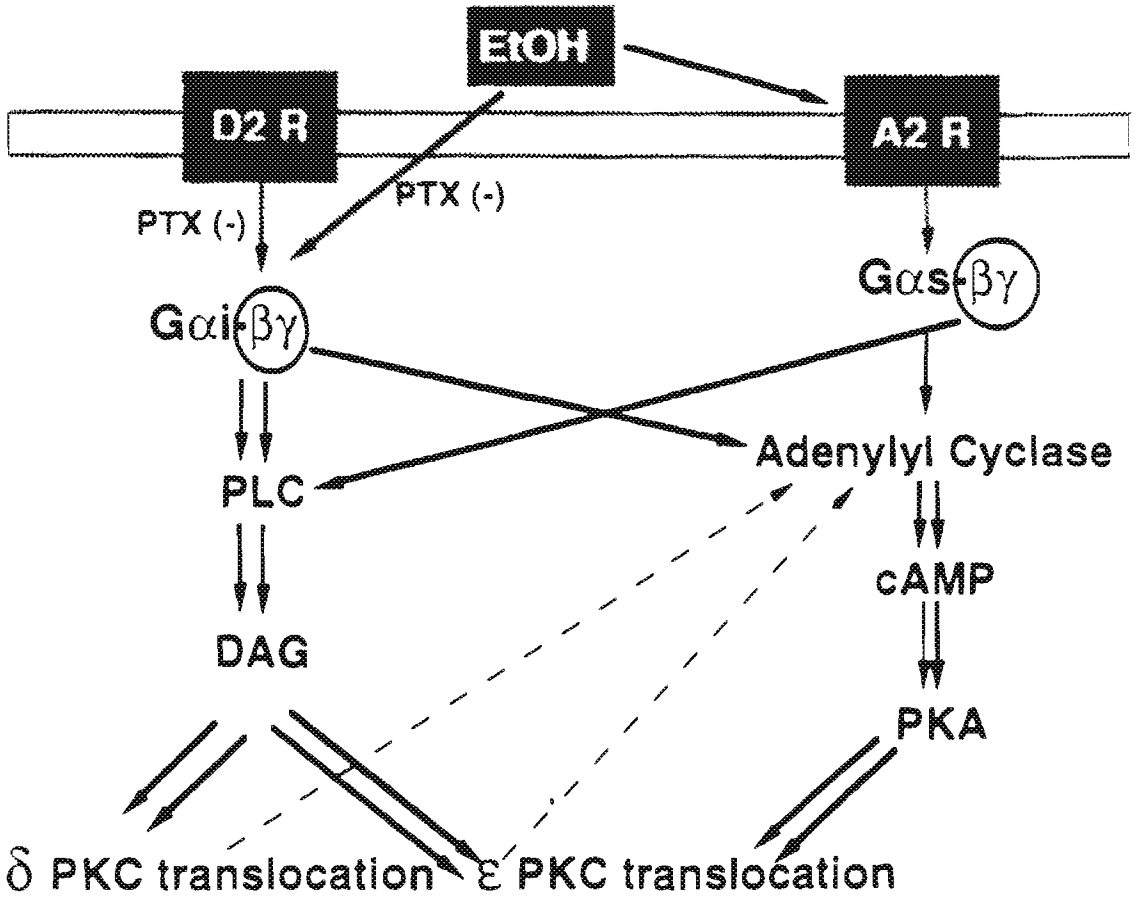

EtOH
D2 R
A2 R
PTX (-)
PTX (-)
Gαi-βγ
Gαs-βγ
Adenylyl Cyclase
PLC
cAMP
DAG
PKA
δ PKC translocation
ε PKC translocation

FIG. 17

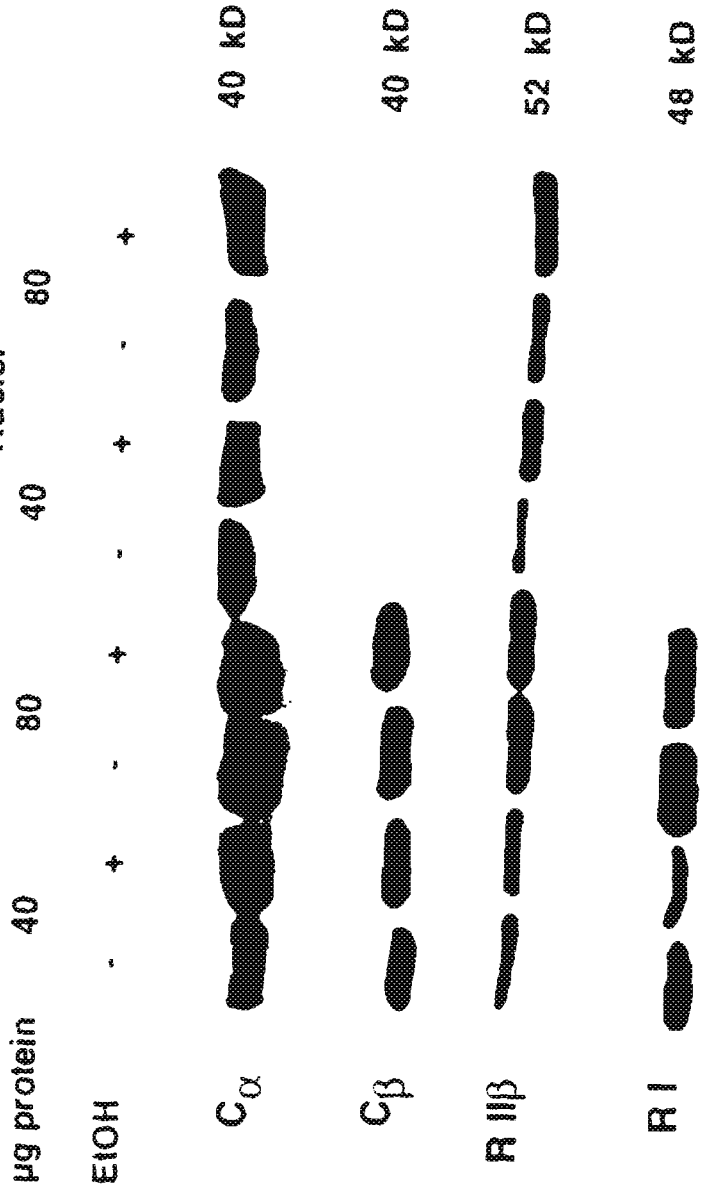

Whole cells
Nuclei
μg protein 40 80 40 80
EtOH - + - + - + - +
Cα
Cβ
R IIβ
R I
40 kD
40 kD
52 kD
48 kD

FIG. 18